Development of Genetic Tools in Methanotrophs and the Molecular Regulation of Methane Monooxygenase

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Abbreviations

$Amp^{(R)}$	ampicillin (resistance)
ATP	adenosine triphosphate
BCCP	bacterial diheme cytochrome c peroxidise
BHR	broad-host range
bp	nucleotide base pairs
2-DE	2-dimensional gel electrophoresis
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxynucleotide triphosphate
E	core RNA polymerase enzyme
EBP	enhancer binding protein
EDTA	ethylendiaminetetraacetic acid
EP-PCR	error-prone PCR
EPR	electron paramagnetic resonance
Eσ	RNA polymerase holoenzyme
FAD	flavin-adenine dinucleotide
g	gram
GFP	green fluorescent protein
$Gm^{(R)}$	gentamicin (resistance)
h	hour
kDa	kilo Daltons
$Km^{(R)}$	kanamycin (resistance)
KO	knock-out
l	litre
LacZ	β-galactosidase
M	molar
MCS	multiple cloning site
MEM	marker-exchange mutagenesis
mg	milligram
min	minute

ml	millilitre
mM	millimolar
mRNA	messenger RNA
MS	mass spectrometry
MUG	4-methylumbelliferyl-β-D-glucuronide
ng	nanogram
NMS	nitrate mineral salts
NRPS	non-ribosomal peptide synthetase
OD_{540}	optical density at 540 nm
ONPG	o-nitrophenol-β-D-galactoside
orf	open reading frame
ori	origin of replication
oriT	origin of transfer
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PKS	polyketide synthetase
PLFA	phospholipid fatty acid
pMMO	particulate methane monooxygenase
RBS	ribosomal binding site
RNase	ribonuclease
rRNA	ribosomal ribonucleic acid
RT-PCR	reverse transcriptase PCR
RuMP	ribulose monophosphate
s	seconds
SD	Shine-Delgarno
SDM	site-directed mutagenesis
SDS	sodium dodecyl sulphate
sMMO	soluble methane monooxygenase
TAE	tris acetate EDTA
TBE	tris borate EDTA
TCE	trichloroethylene
TE	tris EDTA
Tn	transposon
UAS	upstream activating sequence

v/v	volume to volume
w/v	weight to volume
X-gal	5-bromo-4-chloro-3-indoyl-β-D-galactoside
XylE	catechol-2, 3 dioxygenase
µg	microgram
µl	microlitre

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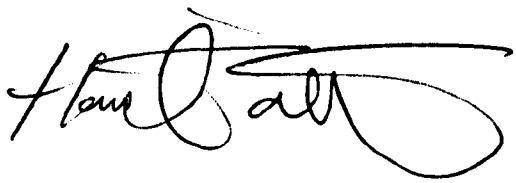
Finally, I would like to acknowledge NERC for funding this studentship and SGM for financial support towards my research visit to Professor Harald Jensen's Lab.

Declaration

I declare that the work presented in this thesis was performed by me under the supervision of Professor J. Colin Murrell and none of the work has been previously submitted for any other degree. Contribution and additional help from others has been specifically acknowledged. Data presented in Chapter 3 has been submitted for publication (**Ali, H., Scanlan, J., Dumont, M. G., & Murrell, J. C. (2006)**. Duplication of *mmoX* gene in *Methylosinus sporium*: Cloning, sequencing and mutational analysis. *Microbiology* **152**, 2931-2942) and some of the data presented in Chapter 5 was published as part of a manuscript (**Theisen, A. R., Ali, M. H., Radajewski, S., Dumont, M. G., Dunfield, P. F., McDonald, I. R., Dedysh, S. N., Miguez, C. B. & Murrell, J. C. (2005).** Regulation of methane oxidation in the facultative methanotroph *Methylocella silvestris* BL2. *Mol Microbiol* **58**, 682-692).



Hanif Ali

Abstract

The oxidation of methane to methanol by methanotrophs is catalysed by the enzyme methane monooxygenase (MMO). In some methanotrophs two distinct MMOs are found; a membrane bound particulate (pMMO) and a cytoplasmic soluble (sMMO) methane monooxygenase. The intracellular location of MMO is dependent on the copper-to-biomass ratio. pMMO is expressed when the copper-to-biomass ratio is high (>0.25 μM) and sMMO is expressed when the copper-to-biomass ratio is low (<0.25 μM). Although a great deal of information is known about the expression of MMO with respect to copper, the molecular mechanism regulating this 'copper-switch' is unknown. The aim of this study was to gain further insights into the regulation of MMO expression by copper ions.

The complete sMMO operon, including the regulatory genes, *mmoR* and *mmoG* were cloned and sequenced from *Methylosinus sporium*. The genes encoding sMMO are present as single copy in methanotrophs. In this study, duplicate copies of the *mmoX* gene encoding for the α-subunit of the hydroxylase were characterised at the molecular and biochemical level. Mutational analysis indicated that the second copy was not essential for sMMO expression and also gave insights into the role of the water soluble pigment in siderophore-mediated iron-acquisition. sMMO-minus mutants were complemented by heterologous expression of sMMO genes from *Methylosinus trichosporium* and *Methylococcus capsulatus*. These experiments demonstrated the amenability of *Ms. sporium* to genetic manipulations facilitating its use as an alternative model organism for molecular analysis of MMO regulation.

To aid transcriptional analysis of the MMO operons, a series of integrative and broad-host range promoter probe vectors, containing *gfp*, *xylE*, km^R or *lacZ*, were constructed and tested with the copper repressible sMMO σ^{54} promoter. The usefulness of these reporter genes for the high-throughput detection of sMMO mutants was also assessed. The expression of LacZ in *Mc. capsulatus* via the sMMO σ^{54} promoter yielded a powerful genetic screen for sMMO mutants. This system was coupled with transposon mutagenesis for surveying the genome on a global scale for sMMO regulatory genes and served as an alternative assay system for detecting sMMO expression. This assay system had the specific advantage in that it was more sensitive and in this context, it was selective for sMMO-minus mutants defective only at the transcriptional level. In collaboration with Robert Csaki (University of Szeged), a transposon mutagenesis protocol was established from which a number of sMMO-minus mutants were identified.

Genetic tools developed in this study were used to investigate copper mediated transcriptional regulation of the pMMO σ^{70} promoter, sMMO σ^{54} promoter and its regulatory genes, *mmoR* and *mmoG*. Transcription of the pMMO operon was shown to be constitutive. The sMMO σ^{54} promoter was reconfirmed to be copper repressible and *mmoR* and *mmoG* were shown to be essential for transcription initiation from the sMMO σ^{54} promoter, but were not regulated themselves by copper at the transcriptional level. All of these data were confirmed by constructing chromosomal gene fusions with various reporter genes, RT-PCR and RNA dot-blotting.

The availability of the *Mc. capsulatus* genome sequence during this study allowed targeted mutagenesis to be carried out on copper targets responsible for copper transport. 'Knock-out' mutants of a copper transporting gene and a non-ribosomal peptide synthetase gene responsible for the putative biosynthesis of methanobactin, which is believed to be involved in delivering copper to pMMO, were constructed. The phenotypes of these mutants with regards to MMO expression are yet to be analysed.

Chapter 1

Introduction

1.1 Introduction

Methanotrophs are a group of Gram-negative bacteria that have the unique ability to grow aerobically using methane as their sole source of carbon and energy (Hanson & Hanson, 1996). They catalyse the oxidation of methane to methanol at ambient temperature and pressure using methane monooxygenase (MMO) (Murrell *et al.*, 2000a). Besides methane, the only other substrate generally utilised by methanotrophs for growth is methanol (Best & Higgins, 1981). There are a few exceptions where strains have been found to utilise methylamine and a narrow selection of other C_1 compounds, but none have been shown to grow on multi-carbon compounds and methanotrophs are generally regarded as obligate for their requirement for methane (Bowman *et al.*, 1993). However, the relatively recent isolation of the facultative methanotroph, *Methylocella silvestris* BL2 changed the perception of methanotrophy as being an obligate trait as it was shown to grow on multi-carbon compounds such as acetate and ethanol (Dunfield *et al.*, 2003; Theisen *et al.*, 2005).

In some methanotrophs, methane is oxidised to methanol by two different MMOs, a membrane bound or particulate MMO (pMMO) and a cytoplasmic or soluble MMO (sMMO) where their expression is dependent on the copper-to-biomass ratio during growth (Prior & Dalton, 1985b; Stanley *et al.*, 1983). The regulation of the expression of MMO formed the basis of this study and in this chapter, a general introduction to the biology of methanotrophic bacteria is given.

1.1.1 Basic characterisation and taxonomy

Bacillus methanicus was the first methane-oxidizing bacterium to be isolated in 1906 by Söhngen. This discovery was largely over-looked until the early 1950s when Foster and colleagues re-isolated similar methane oxidising strains to *Bacillus methanicus*, which was renamed to *Pseudomonas methanica* and another new methane oxidiser, *Methylococcus capsulatus* (Dworkin & Foster, 1956; Foster & Davis, 1966). This sparked-off renewed interests into methane oxidising bacteria. However, it was not until 1970, when the pioneering work carried out by Whittenbury *et al.*, (1970), led to the isolation of more than 100 new gram-negative, aerobic methane-utilising bacteria (Whittenbury *et al.*, 1970). They were separated into five groups, which included *Methylomonas*, *Methylobacter*, *Methylococcus*, *Methylocystis* and

Methylosinus depending largely on morphological type as they possessed intracytoplasmic membranes in two distinct arrangements (Figure 1.1), types of resting stage formed and other physiological and morphological traits.

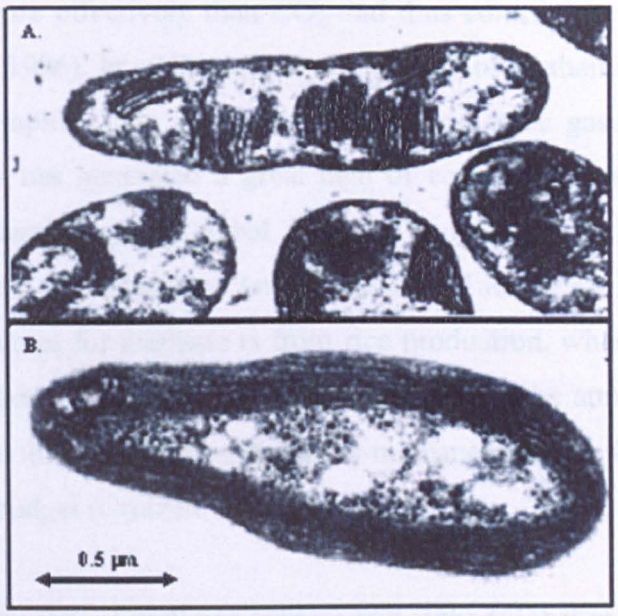


Figure 1.1 Transmission electron micrographs of sections of type I (Panel A) and type II (Panel B) methanotrophs. Type I methanotrophs possess bundles of intracytoplasmic membranes, whereas type II possess intracytoplasmic membranes arranged around the periphery of the cell. Taken from Dalton, (2005).

The current taxonomic scheme for methanotrophs is largely based on the original classification scheme proposed by Whittenbury *et al.*, with the exception of several additional genera which have been grouped into two types, depending on various characteristics (Table 1.1) (Hanson & Hanson, 1996).

Table 1.1 General characteristics of type I and type II methanotrophs. RuMP, ribulose monophosphate pathway; PLFA, phospholipid fatty acid. Adapted from Hanson and Hanson (1996).

Genus name	Type	Characteristics	Type I	Type II
Methylobacter	Type I	G+C content of DNA (mol%)	49-60	62-67
Methylococcus		Membrane arrangement	+	-
Methylocaldum		Bundle of vesicular disks		
Methylomicrobium		Peripheral membrane	-	+
Methylomonas		Resting stages formed	Cyst	Cyst or exopore
Methylosarcina		RuMP pathway present	+	-
Methylosphaera		Serine pathway present	-	+
Methylocystis	Type II	Major PLFAs	16	18
Methylosinus		Proteobacterial subdivision	γ	α
Methylocella				
Methylocapsa				

1.1.2 Ecological significance

Methane is the most abundant organic gas in the atmosphere and absorbs infrared radiation more effectively than CO_2 and thus contributes to global warming (Hanson & Hanson, 1996). In addition, the mixing ratio of methane in the troposphere is increasing more rapidly than any other atmospheric trace gases (Koschorreck & Conrad, 1993). This has generated a great deal of concern and recent attention has been focused on quantifying the global methane budget and the factors leading to increased atmospheric concentrations (summarized in Table 1.2). It can be seen that one of the major sources for methane is from rice production, whereas the major sink is from the degradation of methane by hydroxyl radicals in the atmosphere. However, it has been suggested that microbial oxidation of methane accounts for about 5-10 % of the global methane budget (Crutzen, 1991).

Table 1.2 A summary of methane sources and sinks (global methane budget). All amounts are in Tg/yr (1 Tg = 1 x 10^9 g). Taken from: http://anthonares.net/2006/01/published-research-synopsis-methane.html.

Sources/Sinks	Source Amount	Sink Amount	Net Emission
Animals	80	0	80
Wetlands	142	27	115
Rice Paddies	577	477	100
Biomass Burning	55	0	55
Termites	44	24	20
Landfills	62	22	40
Open waters	85.3	75.3	10
Methane Hydrates	10	5	5
Coal Production	58	18	40
Gas Production	58	18	40
Photochemical Oxidation	0	450	-450
Soil Consumption	0	10	-10

Methanotrophic bacteria are the only known group of bacteria to grow on methane and thus considered to play an important role in regulating atmospheric methane concentrations (Conrad, 1996). They are ubiquitous in the environment and have been isolated from soil, fresh water and marine environments (Bowman *et al.*, 1993; Whittenbury *et al.*, 1970). In a recent study, *Crenothrix polyspora*, a filamentous bacterium present in drinking water was described that had the ability to oxidise methane and contained a phylogenetically unusual particulate methane

monooxygenase gene (Stoecker *et al.*, 2006). The widespread distribution of methanotrophs in diverse environments including hot springs (Bodrossy *et al.*, 1999), saline and alkaline aquatic environments (Trotsenko & Khmelenina, 2002), soda lakes (Lin *et al.*, 2004), sub-arctic peat soils (Dunfield *et al.*, 1993) and acidic peat bogs (Dedysh *et al.*, 1998) further indicates their potential significance in regulating global methane concentrations.

1.1.3 Biotechnological applications

The re-isolation of *Pseudomonas methanica* (Dworkin & Foster, 1956) which initiated a sudden flurry of research activity leading to an appreciation of the basic physiology and biochemistry of methane oxidising bacteria, represents an early example of the influence of renewed interest in the biotechnological applications of methanotrophs. However, over the past 30 years, there has been considerable interest in methanotrophs for their potential use in bioremediation and biotransformation, especially after the discovery that sMMO was capable of oxidising a wide range of substrates that included alkanes, alkenes, alicyclics, aromatic ethers and heterocyclic compounds (Colby *et al.*, 1977). This was a result of a lack of substrate specificity, thus resulting in the fortuitous ability of sMMO to co-oxidise a wide range of compounds. This was attractive for the development of biological methods for bioremediation and for the production of single cell proteins (SCP) and bulk chemicals with high commercial value such as propylene oxide (Smith & Dalton, 2004).

Methanotrophs were found to have the ability to co-oxidise trichloroethylene (TCE) with efficiency at least two fold greater than any other bacteria (Tsien *et al.*, 1989). It has been shown that a type II methanotrophic bacterium, phylogenetically related to *Methylosinus* species was capable of synthesising sMMO and to readily oxidise TCE (Alvarez-Cohen *et al.*, 1992). The current use of methanotrophs for *in-situ* bioremediation of TCE would be ineffective since the sMMO enzyme is largely not expressed due to repression by copper ions. A better understanding of the expression of MMO by copper ions is required, which would make it possible to regulate the expression of sMMO in the environment, thus leading to a greater potential for their exploitation in bioremediation.

The synthesis of enantiopure compounds for the pharmaceutical industry is regarded as very important ever since the thalidomide case, where one form was

administered as a sedative, whereas the other form caused fetal abnormalities during pregnancy (Perri & Hsu, 2003). There are many synthetic routes to produce single enantiomers such as synthesis via resolution of racemates, synthesis from chiral building blocks and synthesis via chiral auxilaries, but they all involve time consuming procedures and importantly they are often not economically feasible for large scale synthesis. However, nature continuously performs stereoselective transformation using enzyme catalysts such as lipase from *Candida rugosa* (Berglund, 2001), which after some modification by site-directed mutagenesis (SDM) improved its enantiomeric selectivity for R-3-hydroxyisobutyric acid (De la Casa *et al.*, 2002), required for the synthesis of D-captopril, an anti-hypertensive drug. This raises the possibility of exploiting the oxidation property of sMMO to increase stereoselectivity. A current research project in the Murrell lab is aimed at exploiting the substrate specificity, in terms of altered activity and stereoselectivity, by SDM of the active site of sMMO.

Despite the recognition of the vast potential of methanotrophs, it was not exploited in an industrial setting until 1994, when Norferm AS was established to commercialise and develop BioProtein technology. At present, Norferm AS is created as a 50/50 joint venture between DuPont and Statoil, where *Methylococcus capsulatus* Bath is used to produce BioProtein, a protein-rich biomass, using methane-rich natural gas as sole nutrients and energy source in a BioProtein plant capable of having an annual production of 8,000 tonnes. The current market for BioProtein or its derivatives are suitable for fish and animal feedstuffs, as a nutritional source and as a functional ingredient. Further research is ongoing for the use in human food, which could open up a large market (www.norferm.com).

Recently the sequencing of the *Mc. capsulatus* genome was carried out jointly by University of Bergen and The Institute for Genomic Research (Ward *et al.*, 2004) and the genome of *Methylomonas* strain 16a has been sequenced by DuPont. The availability of the *Mc. capsulatus* genome sequence will enable us to improve our understanding of the biochemistry of the organism and may enable metabolic engineering of novel pathways incorporating engineered methane monooxygenases for the synthesis of valuable pharmaceuticals and other products from methane and other inexpensive starting materials.

1.2 Methane oxidation pathway

The biochemistry of methane oxidation in methanotrophs have been extensively studied and the general pathway is well established (Figure 1.2) (Anthony, 1982).

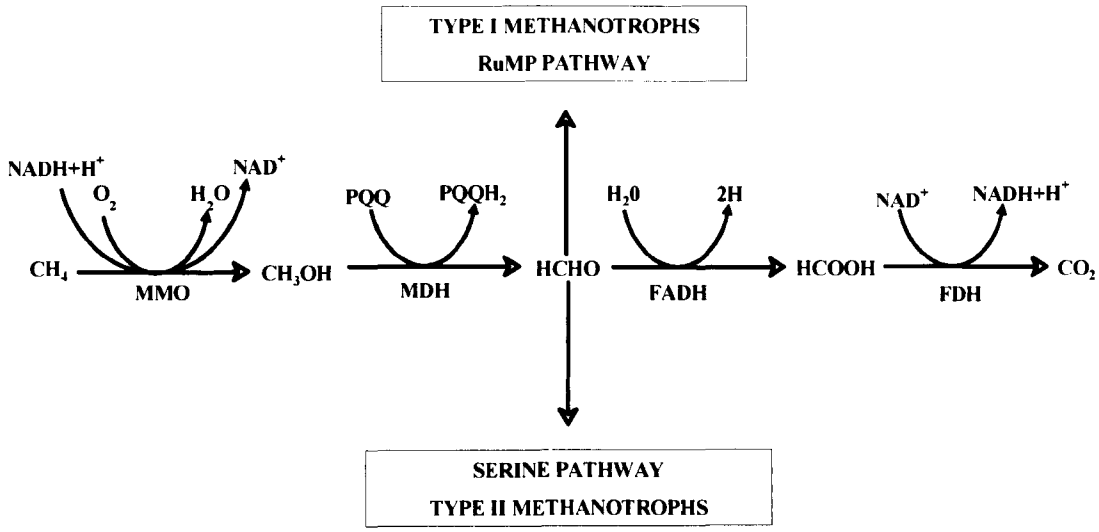


Figure 1.2 Pathway for oxidation of methane and assimilation of formaldehyde.

The first step in methane metabolism is mediated by methane monooxygenase (MMO), which oxidises methane to methanol. This reaction utilises two reducing equivalents to split the O_2 bond of the dioxygen where one of the oxygen atoms is reduced to form H_2O and the other is incorporated into methane to form methanol. Methanol is further oxidised to formaldehyde by methanol dehydrogenase (MDH), which is a periplasmic pyrroloquinoline (PPQ)-dependent enzyme (Anthony, 2004).

Formaldehyde is an important metabolic branch point between dissimilation to produce ATP and assimilation to make new multi-carbon compounds for proteins, DNA and lipids (cell biomass). In methanotrophs there are two major pathways for carbon assimilation. The type I methanotrophs assimilate carbon into cell biomass predominantly via the ribulose monophosphate (RuMP) pathway, whereas the type II methanotrophs use the serine pathway. Approximately 50 % of the carbon is assimilated into cell biomass at the level of formaldehyde and the remaining 50 % is further oxidised to CO_2 via formaldehyde dehydrogenase (FADH) and formate dehydrogenase (FDH). The dissimilatory reaction (oxidation of formaldehyde → CO_2)

is involved in generating reducing equivalents for biosynthesis and initial oxidation of methane. It is noteworthy that in some recent studies, alternative routes for formaldehyde dissimilation have been found in methanotrophs (Vorholt, 2002), which may serve the purpose of formaldehyde detoxification and formaldehyde dissimilation for ATP production.

1.3 Particulate methane monooxygenase (pMMO)

The particulate methane monooxygenase (pMMO) is found in all known methanotrophs with the exception of a recently isolated methanotroph, *Methylocella silvestris* (Dunfield *et al.*, 2003; Theisen *et al.*, 2005). The pMMO has a narrow substrate specificity compared to sMMO, nevertheless, it is capable of oxidising alkanes and alkenes up to five carbons in length, but is unable to oxidise aromatic or alicyclic compounds such as naphthalene and cyclohexane (Burrows *et al.*, 1984).

The synthesis and expression of pMMO are associated with the development of an extensive network of intracytoplasmic membranes, where the membrane-bound pMMO resides, and it is known that addition of copper stimulates the activity of pMMO, both *in-vivo* and *in-vitro* (Prior & Dalton, 1985b; Stanley *et al.*, 1983). It is not known whether the increase in pMMO activity is due to the increase in membrane content or due to the copper ions or both. However, in a study by Semrau *et al.* (1995), electron paramagnetic resonance (EPR) spectroscopy was used to investigate the nature of copper ions associated with membranes and with pMMO activity in *Mc. capsulatus*. The data suggested that the primary role of copper is in the active site of the pMMO rather than simply a structural one (Semrau *et al.*, 1995). Recent studies, which incorporated an improved purification protocol for pMMO (Basu *et al.*, 2003; Lieberman *et al.*, 2003) and the subsequent resolution of the crystal structure of pMMO (Lieberman & Rosenzweig, 2005a), verified the essential role of copper in enzyme catalysis. It is now generally accepted that pMMO is a copper containing protein, which has an obligate requirement for copper, since copper is required for both expression and activity (Nguyen *et al.*, 1994; Nguyen *et al.*, 1998).

1.3.1 Biochemistry of pMMO

The purification of membrane-bound pMMO enzyme has proved to be much more difficult than sMMO because removal from the hydrophobic environment of the lipid bilayer causes the loss of activity, either by altering the native protein conformation or by disrupting critical interactions with other proteins and lipids (Lieberman & Rosenzweig, 2004).

The first reliable purification procedure for the isolation of the pMMO complex from *Mc. capsulatus* was achieved using dodecyl β-D maltoside as the solubilising agent (Smith & Dalton, 1989). Further purification of the protein complex into distinct polypeptides was unsuccessful and resulted in the complete loss of enzyme activity. The subsequent development of improved solubilisation procedures identified the active pMMO complex consisting of two components, the hydroxylase (pMMOH) which comprised of three subunits (α, β, and γ) of approximate masses, 47, 24, and 22 kDa subunits and a putative reductase (pMMOR) comprising of 63 and 8 kDa proteins (Basu *et al.*, 2003; Zahn & DiSpirito, 1996).

The organisation of the three pMMO polypeptides comprising the hydroxylase, the subunit stoichiometry, and the total molecular mass of the holoenzyme are controversial. It was originally assumed that the pMMO polypeptides from *Mc. capsulatus* were present in a 1:1:1 ratio corresponding to a αβγ heterotrimer of ~100 kDa (Yu *et al.*, 2003). An additional report by Lieberman and co-workers (2003), following the active purification of pMMO of a molecular mass of ~200 kDa, suggested that the pMMOH polypeptides are arranged in a $\alpha_2\beta_2\gamma_2$ dimeric configuration (Lieberman *et al.*, 2003). The recent crystallisation of pMMO from *Mc. capsulatus* (2.8 Å resolution) has completely changed our perception of the organisation of the pMMO polypeptides, as three copies each of the αβγ subunits were found to form a cylindrical $\alpha_3\beta_3\gamma_3$ trimer (Figure 1.3) (Lieberman & Rosenzweig, 2005a; Lieberman & Rosenzweig, 2005b). It was interesting to note that two of the three metal centres modelled as mononuclear copper and dinuclear copper, were located in the soluble regions of each α-subunit (PmoB). However, based on previous studies, the β-subunit (PmoA) of the hydroxylase was believed to contain the active site for methane oxidation, as it was shown through binding studies with labelled [^{14}C]-acetylene, a powerful inhibitor of MMO (Cook & Shiemke, 1996; Prior &

Dalton, 1985a). The difference in the location of the copper centers and the binding of acetylene has raised some doubts about the exact location of the active site. The availability of the crystal structure has provided new insights into the molecular details of methane oxidation by pMMO, however, no assessment of the activity of the crystals or biochemical analysis of the oligomeric form was undertaken and it is quite possible, based on their description of the enzyme preparation, that the crystals were of inactive proteins.

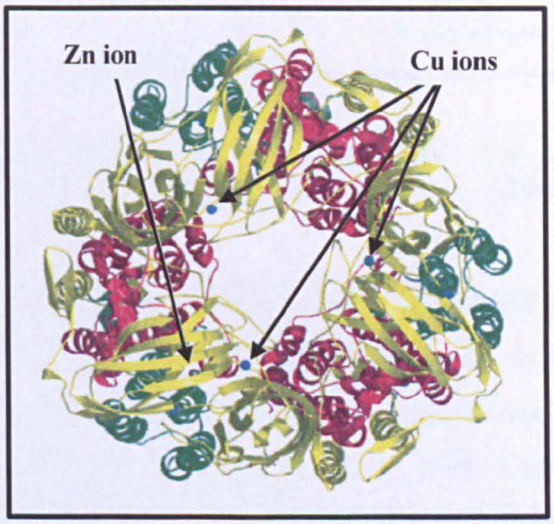


Figure 1.3 The crystal structure of pMMO trimer. The PmoB subunit is shown in yellow, the PmoA subunit is shown in magenta (red), and the PmoC subunit is shown in green. Taken from Lieberman and Rosenzweig (2005a).

In parallel, but in a independent study, Kitmitto and co-workers (2005) described the characterisation of the 3D structure of a highly active form of the pMMOH enzyme, determined by electron microscopy and single-particle analysis at a final resolution of 23 Å (Kitmitto *et al.*, 2005). The biochemical and structural data were in accordance with the recent crystal structure (Lieberman & Rosenzweig, 2005a), which indicated that pMMOH is trimeric, with each monomer unit composed of three polypeptides of 47, 26, and 23 kDa. In addition, side 'holes' in the periplasmically exposed region of the protein were identified, which were proposed to be the areas of potential substrate entry (Figure 1.4).

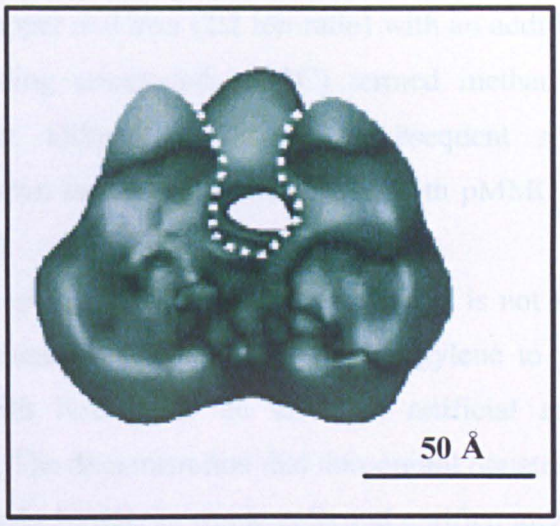


Figure 1.4 The 3D structure of purified pMMOH. The central hole indicates putative site for substrate entry. Taken from Kitmitto *et al.*, (2005).

The pMMO enzyme is generally accepted to be a metalloenzyme, however, the exact metal ion composition of the pMMO active site is the subject of much controversy. All purified pMMO preparations have been shown to contain copper, but varying stoichiometries, ranging from 2 (Basu *et al.*, 2003; Lieberman *et al.*, 2003) to 15 (Takeguchi & Okura, 2000; Yu *et al.*, 2003; Zahn & DiSpirito, 1996) copper ions per αβγ complex have been reported. The reasons for these discrepancies are unclear, but it might be related to procedures for washing the cells and membranes or the methods used to determine the protein concentrations. Lieberman (2005a) recently reported the presence of a mononuclear zinc centre shown in the crystal structure of pMMO (Figure 1.3). However, since there are no other reports of the presence of zinc and the fact that their purified pMMO prior to crystallisation was free of zinc, led to the conclusion that the zinc was derived from zinc acetate, which is present in the crystallisation solution. Thus the physiological presence of zinc remains an open question. In addition, there are disagreements over both the presence and amount of iron. The presence of ~0.5 – 2 iron per αβγ complex have been reported (Basu *et al.*, 2003; Lieberman *et al.*, 2003; Zahn & DiSpirito, 1996), whereas other studies reported the active preparation of pMMO containing no iron (Miyaji *et al.*, 2002; Nguyen *et al.*, 1998; Yu *et al.*, 2003). Interestingly, in an early study by Stanley *et al.*, (1983) the importance of iron for pMMO was demonstrated using ferric and ferrous iron chelators, in the presence of which pMMO activity was severely reduced. In support for the presence of iron, DiSpirito and co-workers have proposed that the catalytic

center contains both copper and iron (2:2 ion ratio) with an additional 6-8 copper ions bound to copper binding compounds (CBC) termed methanobactin, which is a chalkophore (Zahn & DiSpirito, 1996). In subsequent studies, the role of methanobactin was shown *in-vitro* to be associated with pMMO activity (Kim *et al.*, 2004; Kim *et al.*, 2005).

In addition, the physiological source of reductant is not known. However, *in-vitro* pMMO assays, measuring the oxidation of propylene to propylene oxide, are usually performed with NADH or an effective artificial reductant, duroquinol (Shiemke *et al.*, 1995). The demonstration that duroquinol donates electrons directly to pMMO and substitutes for NADH in whole cells and purified membranes opens up the possibility that an *in-vivo* pool of plastoquinol could be a source of reducing equivalents. Despite the significant progress in the past few years towards mechanistic understanding of pMMO, the quest for identifying the active site continues.

1.3.2 Genetics of pMMO

The genes encoding the pMMO have been cloned and sequenced from a number of methanotrophs including *Mc. capsulatus* and *Ms. trichosporium* (Gilbert *et al.*, 2000; Semrau, 1995). In all cases the genes are found to be clustered on the chromosome in the order *pmoCAB* (Figure 1.5).

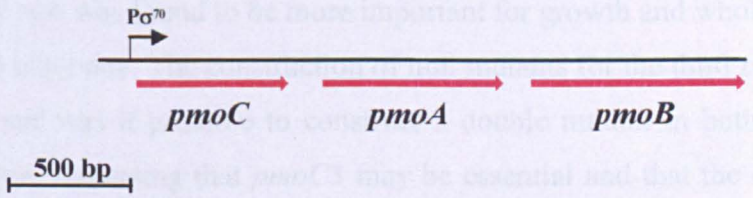


Figure 1.5 The structural gene organisation of pMMO operon in methanotrophs. The *pmoCAB* operon encodes for the γ, β and α subunits of pMMO, respectively.

Initially through hybridisation studies, it was revealed that some methanotrophs contain multiple copies of the genes encoding these pMMO subunits. This was later confirmed by cloning and sequencing of the duplicate copies of the *pmoCAB* operon and a third lone copy of *pmoC* in *Mc. capsulatus* (Stolyar *et al.*, 1999). Sequence analysis revealed that the duplicate copies are virtually identical with only one amino acid difference between the two copies in *Mc. capsulatus* and the copies in

Methylosinus trichosporium OB3b and *Methylocystis* sp. strain M (Gilbert *et al.*, 2000) were also nearly identical. The duplication of the *pmoCAB* genes shows substantial similarity to genes encoding similar subunits of a related enzyme, ammonia monooxygenase (AMO), which are also found in multiple copies and also arranged in the order *amoCAB*. Comparisons of *pmo* and *amo* gene sequences suggest that they could be evolutionarily related (Holmes *et al.*, 1995).

It was shown by Southern hybridisation of DNA from various methane oxidising bacteria isolated from freshwater lake sediment that the number of identical copies varied from one to three in each isolate (Auman *et al.*, 2000). Of the three components of this operon, the *pmoA* gene has been demonstrated to be highly conserved evolutionarily and has been used effectively as a functional marker for surveying methanotrophic diversity in the natural environment (Holmes *et al.*, 1995; McDonald & Murrell, 1997). Using such an approach led to the discovery of novel *pmoA*-like sequences, thus indicating that novel and as yet uncultured species of methanotrophs exist (Henckel *et al.*, 2000; Holmes *et al.*, 1999; Stoecker *et al.*, 2006).

Attempts to characterise the duplicate copies were made by constructing chromosomal insertion mutations in all seven genes of pMMO in *Mc. capsulatus* (Stolyar *et al.*, 1999). The subsequent mutant analysis revealed that the two-*pmoCAB* copies were functionally equivalent and thus both play a role in oxidising methane to methanol. Neither copy of the *pmoCAB* operon was found to be absolutely essential, however, copy two was found to be more important for growth and whole cell methane oxidation than copy one. The construction of null mutants for the third *pmoC* gene was not possible, nor was it possible to construct a double mutant in both copies of the *pmoCAB* operon, indicating that *pmoC*3 may be essential and that the cell requires at least one copy for normal growth.

Transcriptional analysis of the pMMO promoters by primer extension analysis indicate that the *pmoCAB* gene clusters are transcribed from a single transcriptional start site located 300 bp upstream of the *pmoC*1 gene for *Methylocystis* sp. Strain M (Gilbert *et al.*, 2000) and 135 bp and 132 bp upstream of *pmoC*1 and *pmoC*2 in *Mc. capsulatus* (Stolyar *et al.*, 2001). In both cases, immediately upstream of the putative start site, a consensus sequence for σ^{70} promoters was identified. Similar consensus sequences for the σ^{70} promoter can be found upstream of *pmoC* in other organisms. The pMMO genes of *Mc. capsulatus* has been shown by Northern blot analysis to be transcribed into a polycistronic mRNA of 3.3 kb (Nielsen *et al.*, 1997). However, the

transcriptional regulation of *pmoCAB* operon by copper ions is unclear and is discussed in Section 1.6.

1.4 Soluble methane monooxygenase (sMMO)

The soluble methane monooxygenase (sMMO) unlike pMMO, is cytoplasmic in location, expressed when copper is limiting for growth and only found in some methanotrophs (Murrell *et al.*, 2000a). Thus sMMO can be viewed as an alternative methane monooxygenase, which may provide competitive advantage to those methanotrophs that only contain pMMO as they will be able to colonise a wider range of habitats. It is noteworthy that the phylogenetic distribution of soluble monooxygenases is scattered amongst methanotrophic genera and are considered to be 'homeless' enzymes since they are not maintained permanently in a particular bacterial lineage (Leahy *et al.*, 2003).

Apart from the unique ability of sMMO to oxidise methane to methanol under ambient conditions, it has the remarkable ability to oxidise a wide range of non-growth substrates, which include alkanes, alkenes and aromatic compounds including naphthalene, making it an attractive enzyme for its exploitation in biotechnology (Sullivan *et al.*, 1998). It is noteworthy that despite the lack of substrate specificity, it has been shown through kinetic studies of sMMO that methane is oxidised more efficiently than any other alkanes (Green & Dalton, 1986). A comprehensive list of the substrates sMMO is capable of oxidising can be found in a recent review by Smith and Dalton, (2004).

1.4.1 Biochemistry of sMMO

The cytoplasmic location and the stability of the sMMO enzyme allowed its purification from several methanotrophs (Fox *et al.*, 1989; Pilkington & Dalton, 1991; Woodland & Dalton, 1984).

The sMMO enzyme is a multi-component enzyme. It utilises dioxygen to catalyse the initial hydroxylation step in the pathway for methane oxidation. It is known that sMMO consists of three-components: a hydroxylase protein composed of three subunits arranged in a $(\alpha\beta\gamma)_2$ configuration in which the α, β and γ subunits have masses of 61, 45 and 20 kDa, respectively; a small effector or coupling protein, protein

B (MmoB), which has a mass of 16 kDa and a 39 kDa NADH-dependent reducatse (MmoC) (Figure 1.6).

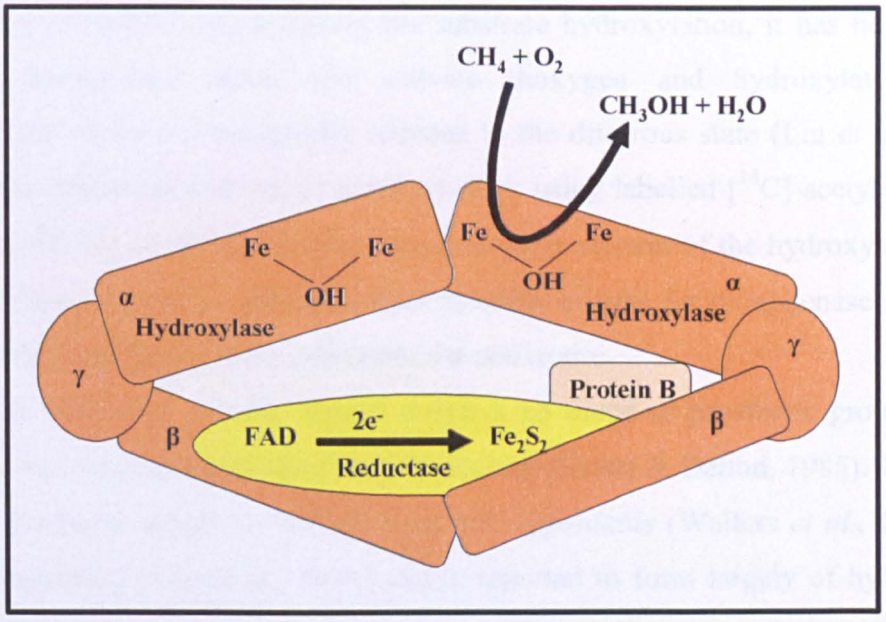


Figure 1.6 Schematic representation of the multi-component soluble methane monooxygenase enzyme complex.

The crystal structure of the hydroxylase components of sMMO has been solved from *Mc. capsulatus* (Rosenzweig *et al.*, 1993) and *Ms. trichosporium* (Elango *et al.*, 1997) to 1.7 Å and 2 Å, respectively (Figure 1. 7).

Figure 1.7 Model structure of the hydroxylase. The α-subunit is shown in blue, β-subunit in green and γ-subunit in orange. The binuclear iron centres are represented by two red spheres on the α-subunits. Taken from Rosenzweig *et al.*, (1993).

Structural analysis confirmed that the α-subunit of the hydroxylase contains a μ-hydroxo-bridged binuclear iron center (Green & Dalton, 1988). Although the three components of sMMO are necessary for substrate hydroxylation, it has been shown that the hydroxylase alone can activate dioxygen and hydroxylate various hydrocarbons when it is chemically reduced to the diferrous state (Liu *et al.*, 1997). These observations, in addition to previous work using labelled [^{14}C]-acetylene (Prior & Dalton, 1985a), which specifically bound to the α-subunit of the hydroxylase, gives strong evidence that this region is almost certainly critical for dioxygenase activation and substrate binding and thus constitutes the active site.

The regulatory protein, MmoB contains no metal or prosthetic groups and is thought to be a strong regulator of sMMO activity (Green & Dalton, 1985). The NMR structure has been solved for MmoB from *Mc. capsulatus* (Walters *et al.*, 1999) and *Ms. trichosporium* (Fox *et al.*, 1991) and is reported to form largely of hydrophobic surface suitable for binding the hydrophobic cleft formed at the $\alpha_2\beta_2$ interface of the hydroxylase, where it is believed to induce conformational changes to the hydroxylase upon binding (Wallar & Lipscomb, 1996). In addition, it has been shown that MmoB enhances the rate of electron transfer between the reductase and the hydroxylase (Froland *et al.*, 1992). However, detail of the exact mechanism of how the cofactorless MmoB protein regulates various aspects of MMO catalysis remains unclear.

The third component, MmoC, contains one [2Fe-2S] cluster and one flavin adenine dinucleotide (FAD) cofactor, which are involved in transferring electrons from NADH to the diiron site of the hydroxylase (Lund *et al.*, 1985a; Lund & Dalton, 1985b). Similar to MmoB, it has been shown that MmoC interacts with the hydroxylase (Fox *et al.*, 1991). In addition, it has been shown that copper ions are a potent inhibitor of MmoC and thus sMMO activity. Copper was found to exert its effect primarily at the iron-sulphur centre, which subsequently disrupts the protein structure, preventing the transfer of electrons from NADH to the hydroxylase (Green *et al.*, 1985). It is noteworthy that in this experiment, 10 mM $CuSO_4$ were used in whole cells and 80 μM in MMO assay on cell extracts. In cell extracts, no activity was observed in the presence of copper, however, in whole cells almost a quarter of the wild-type sMMO activity was observed even in the presence of 10 mM $CuSO_4$ (Green *et al.*, 1985). This gives hope of engineering a methanotrophic strain capable of

expressing sMMO constitutively and without being too concerned about copper inhibiting sMMO through MmoC (Protein C).

In addition to the structural and biochemical aspects of sMMO systems, several aspects of the catalysis, including dioxygen activation and substrate hydroxylation at the active site has been studied extensively in *Mc. capsulatus* and *Ms. trichosporium* and has been reviewed by several authors (Lipscomb, 1994; Merkx *et al.*, 2001; Wallar & Lipscomb, 1996).

Attempts to express sMMO from *Mc. capsulatus* in *E. coli* were made, but only active expression of MmoB and MmoC was possible. The expression of recombinant hydroxylase in *E. coli* was inactive and since the hydroxylase forms a complex structure itself, it was thought that the necessary assembly proteins were lacking in this heterologous host (West *et al.*, 1992). However, the active expression of protein B in *E. coli* allowed site-directed mutagenesis to be carried out to investigate the N-terminal cleavage of MmoB at specific amino-acyl residues, which resulted in the formation of an inactive MmoB protein due to a conformational change, preventing MmoB from binding the hydroxylase (Gallagher *et al.*, 1999). In *Mc. capsulatus* the N-terminal sequence contains Met-12 and Gly-13, however, in *Ms. trichosporium*, the N-terminal sequence contains Met-12 and Gln-13, but similar N-terminal cleavage did not take place. Therefore by substitution of Gly-13 with Gln-13 dramatically stabilised the activity of this polypeptide in *Mc. capsulatus* (Lloyd *et al.*, 1997).

The heterologous expression of sMMO was first demonstrated by Lloyd *et al.* in methanotrophic strains that do not normally express sMMO (Lloyd *et al.*, 1999b). The sMMO structural genes from *Ms. trichosporium* was expressed in *Methylomicrobium album* BG8 and *Methylocystis parvus* OBBP from a broad-host range plasmid containing the structural genes encoding sMMO, its native promoter together with the regulatory genes, *mmoR* and *mmoG* (discussed in Section 1.6). Using a similar expression system, it was possible to complement the sMMO-minus phenotype of *Ms. trichosporium* mutant strain (Lloyd *et al.*, 1999a)

1.4.2 Genetics of sMMO

The complete set of structural genes encoding sMMO were first cloned and sequenced from *Mc. capsulatus* (Stainthorpe *et al.*, 1989; Stainthorpe *et al.*, 1990). This led to the cloning and sequencing of sMMO structural genes from a number of methanotrophs (Figure 1.8) including *Ms. sporium*, which was cloned and sequenced as part of this study (Chapter 3). In all cases, the organisation of the structural genes is found in the order *mmoXYBZDC*. Unlike pMMO, the genes encoding sMMO are present as a single copy in the organisms examined, however in this study, identification of duplicate copies of *mmoX* gene in *Ms. sporium* is reported (Chapter 3).

It was demonstrated by Northern blotting that these sMMO genes constitute a single operon (Nielsen *et al.*, 1996). The *mmoX*, *mmoY* and *mmoZ* encode for the α, β and γ-subunits of the hydroxylase and *mmoB* and *mmoC* encode for the regulatory protein, MmoB and the reductase, MmoC, respectively. The *mmoD* gene encodes for MmoD, a protein of unknown function. However, in a recent study in *Mc. capsulatus*, MmoD was shown to be expressed at low levels and to interact with the hydroxylase and therefore thought to play a regulatory role (Merkx & Lippard, 2002). Furthermore, initial attempts to express sMMO in the absence of *mmoD* was not possible, suggesting that MmoD is required for expression (Dumont, 2004).

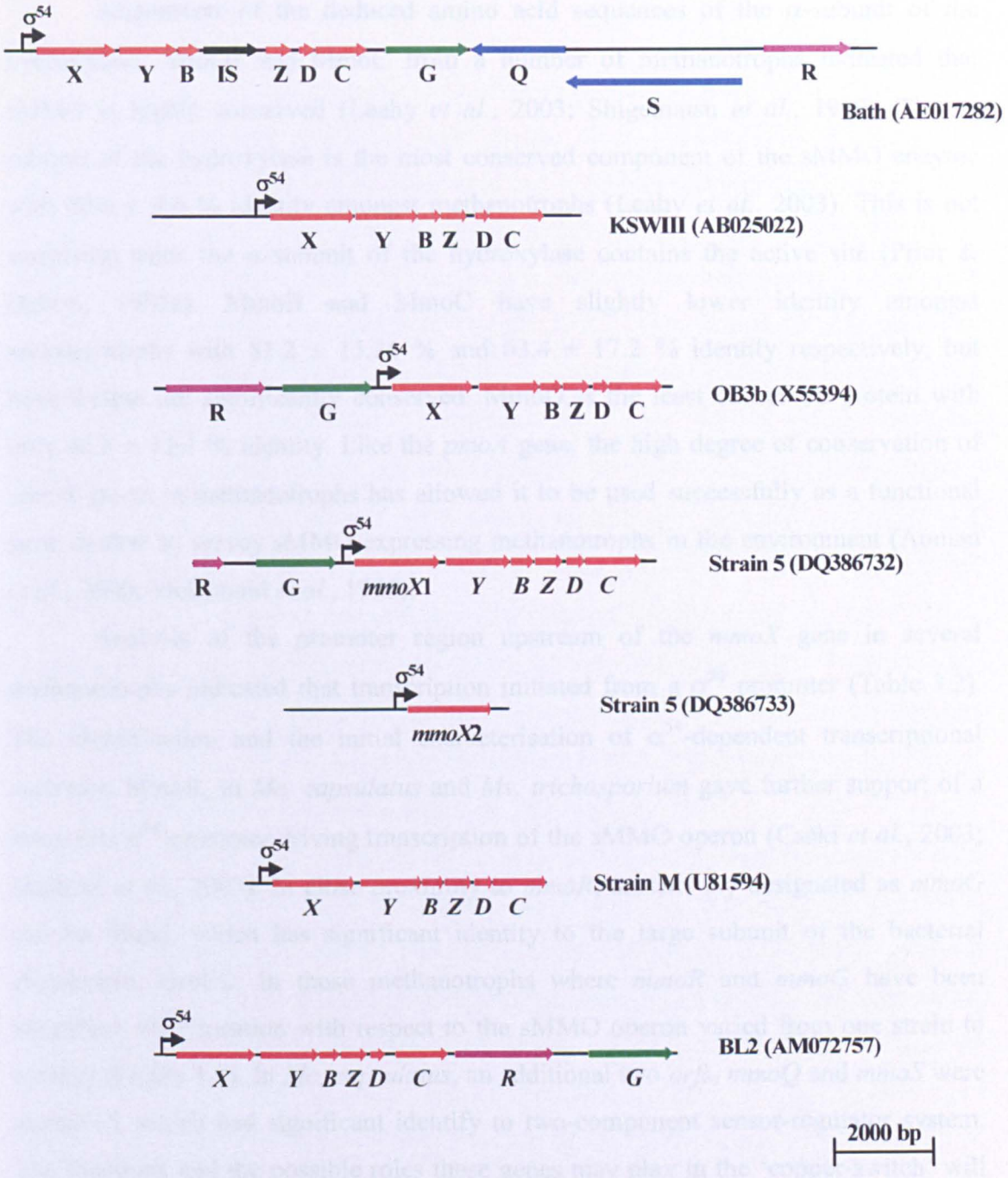


Figure 1.8 Physical maps of the sMMO operons from a number of type I and type II methanotrophs. The genes encoding for the sMMO enzyme (*mmoXYBZDC*) are highlighted in red. The regulatory genes, *mmoG* and *mmoR* are highlighted in green and purple respectively. The insertion sequence (IS) located between *mmoB* and *mmoZ* in *Mc. capsulatus* is highlighted in black. This IS element was not present in the original sequence of *Mc. capsulatus* (M90050) or present in any other methanotrophs. The genes encoding for a two-component senor-regulatory system are highlighted in blue. The GenBank accession numbers for the nucleotide sequences of the sMMO operons are indicated in parentheses. The location and direction of transcription from the σ^{54} promoter is indicated with a black arrow. The gene boundaries are shown to scale and are indicated by the scale bars.

Alignments of the deduced amino acid sequences of the α-subunit of the hydroxylase, MmoB and MmoC from a number of methanotrophs indicated that sMMO is highly conserved (Leahy *et al.*, 2003; Shigematsu *et al.*, 1999). The α-subunit of the hydroxylase is the most conserved component of the sMMO enzyme with 86.6 ± 9.6 % identity amongst methanotrophs (Leahy *et al.*, 2003). This is not surprising since the α-subunit of the hydroxylase contains the active site (Prior & Dalton, 1985a). MmoB and MmoC have slightly lower identity amongst methanotrophs with 81.2 ± 15.25 % and 63.4 ± 17.2 % identity respectively, but nevertheless are significantly conserved. MmoD is the least conserved protein with only 46.8 ± 12.1 % identity. Like the *pmoA* gene, the high degree of conservation of *mmoX* genes in methanotrophs has allowed it to be used successfully as a functional gene marker to survey sMMO expressing methanotrophs in the environment (Auman *et al.*, 2000; McDonald *et al.*, 1995).

Analysis of the promoter region upstream of the *mmoX* gene in several methanotrophs indicated that transcription initiated from a σ^{54} promoter (Table 3.2). The identification and the initial characterisation of σ^{54}-dependent transcriptional activator, MmoR, in *Mc. capsulatus* and *Ms. trichosporium* gave further support of a bona fide σ^{54} promoter driving transcription of the sMMO operon (Csaki *et al.*, 2003; Stafford *et al.*, 2003). In close proximity to *mmoR*, another *orf* designated as *mmoG* can be found, which has significant identity to the large subunit of the bacterial chaperonin, GroEL. In those methanotrophs where *mmoR* and *mmoG* have been identified, their location with respect to the sMMO operon varied from one strain to another (Figure 1.8). In *Mc. capsulatus*, an additional two *orf*s, *mmoQ* and *mmoS* were identified, which had significant identify to two-component sensor-regulator system. The functions and the possible roles these genes may play in the 'copper-switch' will be discussed in Section 1.6.

1.5 Genetic systems in methanotrophs

Due to difficulties in isolating mutants of methanotrophs, much of the earlier work utilised chemical mutagens such as the suicide substrate dichloromethane (DCM), UV light and gamma radiation to generate mutants. This was primarily due to the obligate nature of methanotrophs for methane, the lack of suitable plasmids as cloning vectors and the limited systems for introducing and maintaining heterologous vectors in these organisms(reviewed in Murrell, 1992; 1994). It was not until the work of Martin and Murrell, (1995) that a suitable technique was established for generating stable mutants by marker-exchange mutagenesis (Martin & Murrell, 1995). Using this technique, mutant F was generated, where *mmoX*, the gene encoding the α-subunit of the hydroxylase was inactivated. This technique employed a suicide plasmid, into which two regions flanking the gene of interest were cloned. In between these regions an antibiotic marker was cloned and using the conjugal transfer system of *E. coli* S17.1 λ*pir*, targeted genes are 'knocked-out' following a double homologous recombination event. Based on this system for generating stable mutants, a number of targeted mutants were generated in this study, in addition to the development of a number of promoter probe vectors, which are discussed fully in Chapter 4.

Many of the molecular genetic tools available for methanotrophs have been designed based on sMMO due to its amenability to genetic manipulation, its relative stability and ease of purification. The use of broad-host range (BHR) plasmids based on Inc P1 plasmids was demonstrated to be most successful in methanotrophs (McPheat *et al.*, 1987) and based on these plasmids, Lloyd and co-workers demonstrated the heterologous and homologous expression of sMMO in methanotrophs containing only pMMO (Lloyd *et al.*, 1999a; Lloyd *et al.*, 1999b). In addition, Smith and co-workers elegantly developed a genetic system for protein engineering of the hydroxylase component of sMMO in *Ms. trichosporium* thus allowing facile cloning of mutagenized copies of *mmoX* and allowing their subsequent introduction into mutant F (Smith *et al.*, 2002). This system is limited to the α-subunit since the expression of the genes encoding the β and γ subunits of the hydroxylase is unaffected (Lloyd *et al.*, 1999b). A new system has been recently developed in the Murrell Lab by Marc Dumont based on *Ms. trichosporium* SMDM strain which has all the sMMO structural genes deleted or disrupted thus allowing the heterologous expression of recombinant protein of all the components of sMMO (Dumont, 2004).

1.6 The 'copper-switch' and the transcriptional regulation of MMO operons

The intracellular location of MMO activity in methanotrophs that possess both sMMO and pMMO was first demonstrated in chemostat cultures of *Mc. capsulatus* and *Ms. trichosporium* to be dependent on the copper-to-biomass ratio (Prior & Dalton, 1985b; Stanley *et al.*, 1983). They reported the switch from sMMO to pMMO expression, which was accompanied by the development of extensive intracytoplasmic membranes, following the addition of copper. Despite this key finding, little was known about the regulation of the gene clusters for both sMMO (*mmoXYBZYC*) and pMMO (*pmoCAB*) and how copper ions, ultimately regulated the expression of MMO. This led to a number of studies exploring the regulation of this 'copper-switch'.

The first comprehensive study investigating the 'copper-switch' at the transcriptional level was carried out by Nielson and co-workers (Nielsen *et al.*, 1996; Nielsen *et al.*, 1997). From this study it was shown that the transcription of the sMMO gene cluster occurred from a σ^{54} promoter located 5' of *mmoX* and that transcription was negatively regulated by copper ions since no transcripts for sMMO were detected 15 min after the addition of copper to a steady-state chemostat cultures of *Mc. capsulatus* expressing sMMO. In addition, it was also reported from the same study that the synthesis of mRNA for pMMO genes were activated by copper, whereas, under low copper or sMMO expressing conditions, few or no transcripts for pMMO were detected. Although the exact mechanism of the copper-dependent regulation of the sMMO and pMMO gene clusters was not clear, based on experimental evidence on the regulation of MMO for *Ms. trichosporium*, a hypothetical model on the regulation of MMO was proposed (Murrell *et al.*, 2000b) (Figure 1.9).

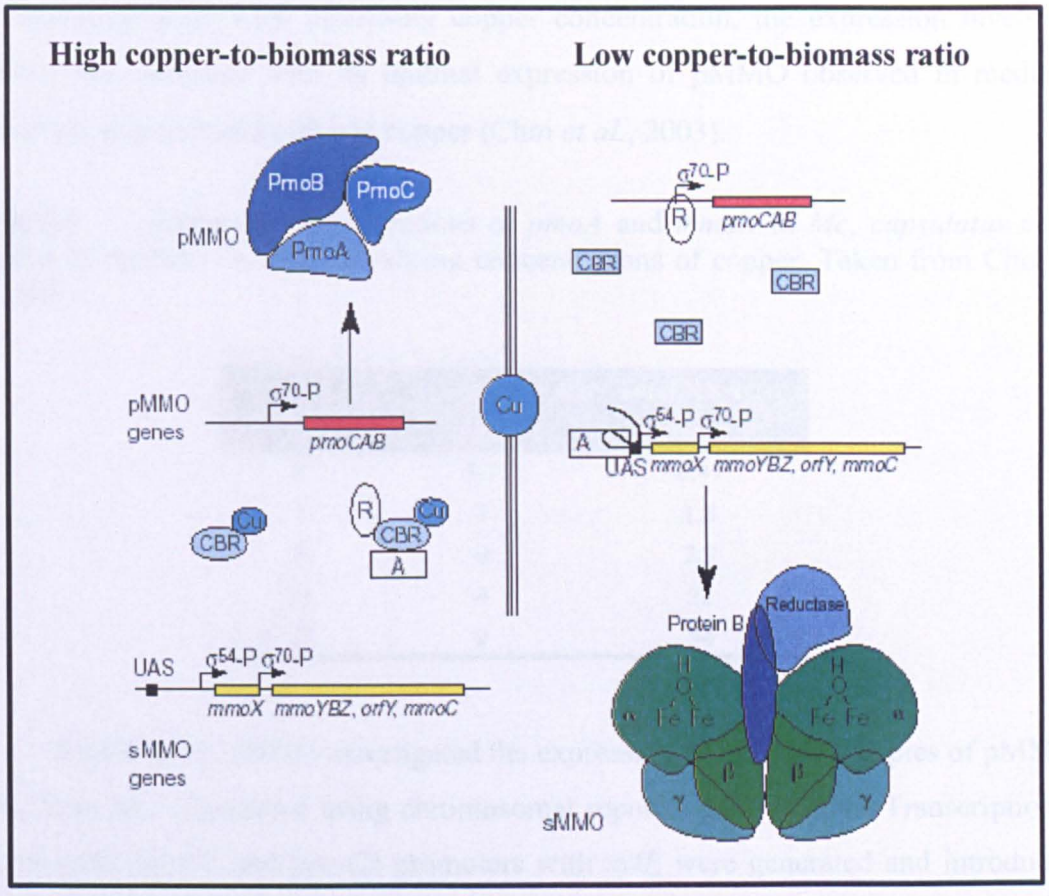


Figure 1.9 Hypothetical model of MMO regulation in *Ms. trichosporium*. Under high copper-to-biomass ratio, pMMO is expressed, whereas, under low copper-to-biomass-ratio sMMO is expressed. In this model, the regulators; repressor (R), activator (A), copper binding regulator (CBR), and the upstream activating sequence (UAS) are all hypothetical. When copper binds to CBR under high copper–to-biomass ratio, the conformation change takes place allowing it to bind R and A, thus preventing R from repressing pMMO gene and A from activating sMMO gene. Under low copper-to-biomass ratio, CBR is unable to form a complex with R or A and therefore R represses pMMO gene and A activates sMMO gene by binding to the UAS. Taken from Murrell *et al.*, (2000)

In a separate study by Choi *et al.* (2003), quantitative RT-PCR was used to measure the amount of *mmoX* and *pmoA* transcripts in *Mc. capsulatus* cultures grown under varying copper concentrations (data summarised in Table 1.3) (Choi *et al.*, 2003). These data confirmed the negative transcriptional regulation of the sMMO operon by copper ions. However, transcripts for *pmoA* were detected from cultures grown with no added copper and when sMMO was fully expressed suggesting that the MMO operons are regulated independently. In addition, these results showed that the level of *pmoA* transcripts increased with increasing concentrations of copper. This was

not surprising since with increasing copper concentration, the expression levels of pMMO also increased with an optimal expression of pMMO observed in medium containing approximately 60 μM copper (Choi *et al.*, 2003).

Table 1.3 Transcript concentrations of *pmoA* and *mmoX* in *Mc. capsulatus* cells cultured in medium containing varying concentrations of copper. Taken from Choi *et al.*, 2003.

[Cu]/μM	mol mRNA/μg Total RNA	
	mmoX	*pmoA*
0	3.1	0.47
1	0	1.8
5	0	2.9
25	0	22
55	0	34

Stolyar *et al.*, (2001) investigated the expression of individual copies of pMMO genes from *Mc. capsulatus* using chromosomal reporter gene fusions. Transcriptional fusions with *pmoC*1 and *pmoC*2 promoters with *xylE* were generated and introduced into the chromosome by conjugation and the XylE activities measured from copper-depleted medium and medium containing 20 μM copper (data summarised in Table 1.4).

Table 1.4 XylE activity of transcriptional fusions of *pmoC* in *Mc. capsulatus* cells grown under high and low copper conditions. Taken from Stolyar *et al.*, 2001.

Promoter	[copper]/μM	XylE activity (nmol/min/mg of protein)/ hrs					
		0	2	4	6	12	24
pC1	1	1.0	8.0	13.0	13.5	32.0	65.0
	20	1.0	4.1	7.0	13.7	52.0	106.0
pC2	0	1.0	19.0	20.9	58.0	60.0	75.0
	20	1.0	18.1	18.2	41.0	75.0	131.0

These data varied from those reported by Nielsen, where few or no pMMO transcripts were detected under copper depleted conditions and also from data reported by Choi, where more than a 40 fold increase in *pmoA* transcripts was detected at 25 μM copper concentrations. These data have created considerable confusion over the exact

transcriptional regulation of the pMMO operon and the concomitant transcription of the sMMO operon.

The regulatory genes for sMMO, *mmoR* and *mmoG* have been cloned and characterised in *Ms. trichosporium* and *Mc. capsulatus* (Csaki *et al.*, 2003; Stafford *et al.*, 2003). MmoR is a σ^{54}-dependent transcriptional activator and to demonstrate its role in sMMO expression, the gene was disrupted by marker-exchange mutagenesis. This resulted in a mutant strain designated JS1, which was unable to express sMMO. MmoG, which encodes for a GroEL-like chaperonin, whose exact function is unclear, was also disrupted by marker-exchange mutagenesis, creating a mutant strain designated JS2. Mutant strain JS2 was also unable to express sMMO and thus it was proposed it may be a MmoR-specific chaperone involved in the assembly of the transcriptional complex.

Based on sequence information, it has been shown that the transcription of the sMMO operon is initiated from a σ^{54} promoter. This was confirmed experimentally by Stafford *et al.*, (2003) by disrupting the *rpoN* gene, which encodes for the σ-factor, σ^{54}, required by RNA polymerase to initiate transcription from σ^{54} promoters. This generated a mutant strain designated Gm1, which was unable to initiate transcription of the sMMO operon (Figure 1.10). From this study, it was proposed that a functional sMMO enzyme complex require the transcriptional factors, MmoR, RpoN as well as the putative MmoR-specific chaperone, MmoG and an improved model of the transcriptional regulation of sMMO expression was proposed (Figure 1.10) (Stafford *et al.*, 2003).

In *Mc. capsulatus*, similar 'knock-out' mutants of *mmoR* and *mmoG* were constructed, which also resulted in sMMO minus mutants (Csaki *et al.*, 2003). In addition, genes encoding a two-component sensor-regulator, *mmoQ* and *mmoS*, were identified 3' of the sMMO operon. Due to their close proximity to the sMMO operon, it was assumed that they may regulate the transcription of the sMMO operon and an alternative hypothetical model was proposed (Figure 1.11) (Csaki *et al.*, 2003).

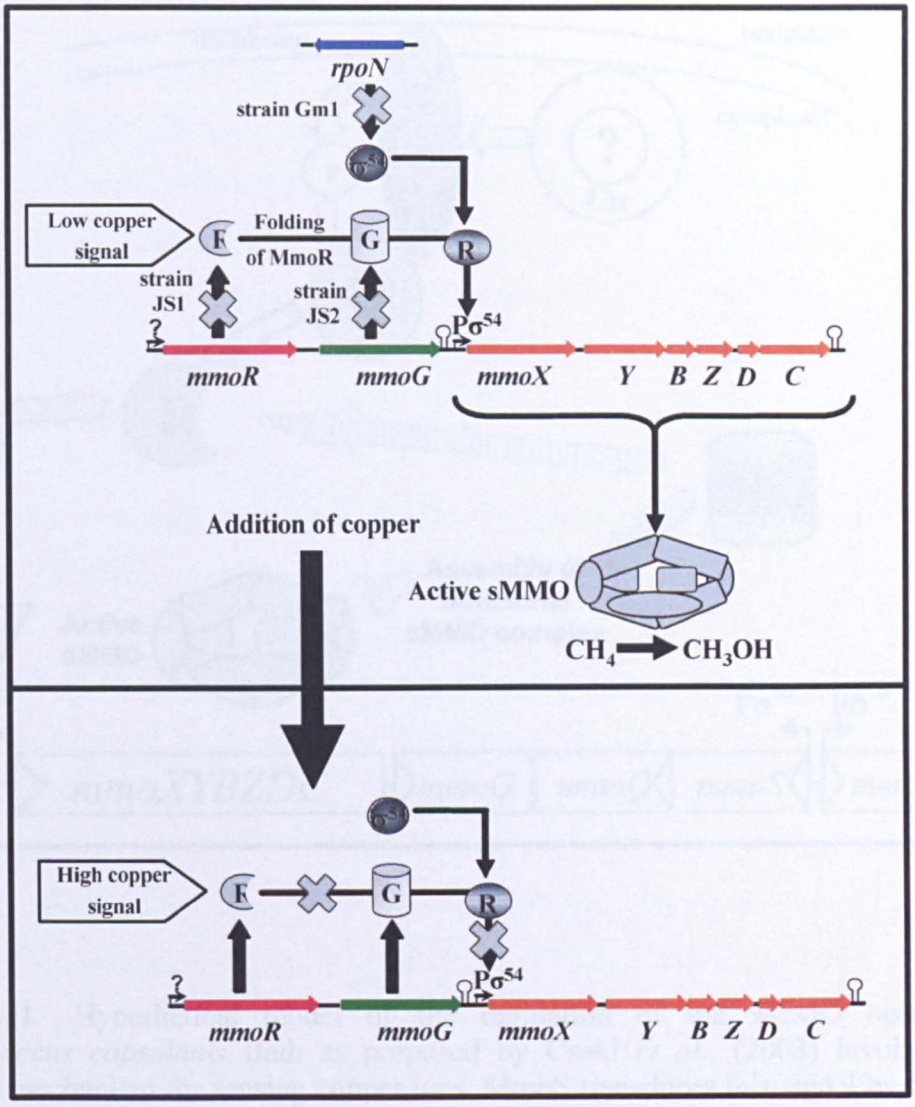


Figure 1.10 Hypothetical model of the regulation of the sMMO operon in *Methylosinus trichosporium* OB3b as proposed by Stafford *et al.*, (2003). The transcriptional regulation of the sMMO operon is regulated by copper ions by an as yet unknown mechanism. Both MmoR (R) and MmoG (G) are required for activating the transcription of the sMMO operon. MmoR in conjunction with σ^{54} is required for transcription. MmoG may be responsible for the correct folding of MmoR or other polypeptide involved in activating MmoR. Inactivation of RpoN (strain Gm1), MmoR (strain JS1) or MmoG (strain JS2) abolishes sMMO expression. In the presence of high copper, transcriptional activation mediated by MmoR and σ^{54} is abolished. Taken from Stafford *et al.*, (2003).

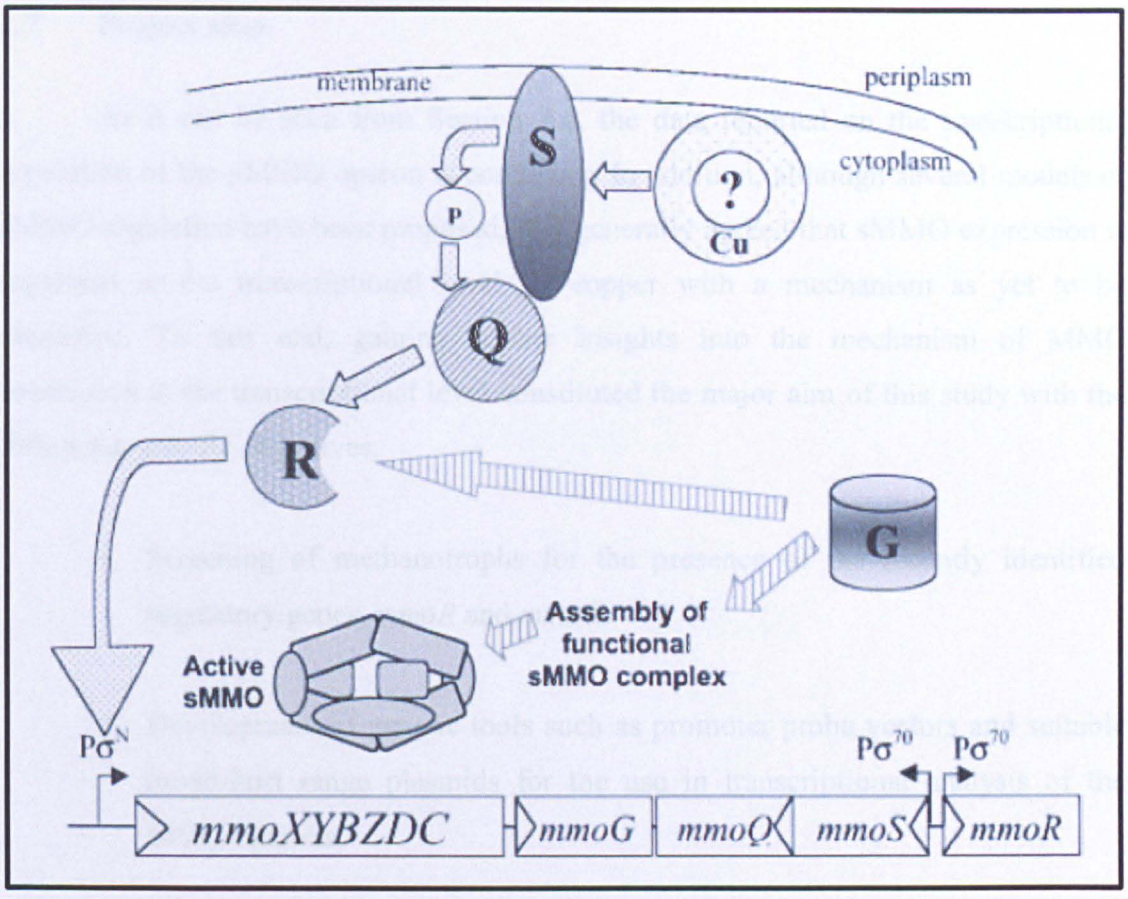


Figure 1.11 Hypothetical model of the regulation of the sMMO operon in *Methylococcus capsulatus* Bath as proposed by Csaki *et al.*, (2003) involving an unknown mechanism for sensing copper ions. MmoS transduces this signal by altering the phosphorylation state of MmoQ. This signal is subsequently relayed to MmoR, which activates the transcription of the sMMO operon via σ^{54}. The exact function of MmoG is unknown, however, it can be assumed that it plays a role in the correct folding of MmoR and/or for the maturation of active sMMO. Taken from Csaki *et al.*, (2003).

1.7 Project aims

As it can be seen from Section 1.6, the data reported on the transcriptional regulation of the pMMO operon is confusing. In addition, although several models of sMMO regulation have been proposed, it is generally agreed that sMMO expression is regulated at the transcriptional level by copper with a mechanism as yet to be identified. To this end, gaining further insights into the mechanism of MMO expression at the transcriptional level constituted the major aim of this study with the following specific objectives:

1. Screening of methanotrophs for the presence of the recently identified regulatory genes, *mmoR* and *mmoG*.

2. Development of genetic tools such as promoter probe vectors and suitable broad-host range plasmids for the use in transcriptional analysis of the MMO operons.

3. Development of a genetic screen for the high-throughput detection of MMO mutants for the use in transposon mutagenesis experiments.

4. Using above tools for identifying and characterising additional as of yet unidentified MMO regulatory genes involved in transcriptional regulation of the MMO operons.

Chapter 2

Materials and Methods

2.1 Bacterial strains and plasmids

Chemically competent *E. coli* TOP10F cells were generally transformed in order to facilitate genetic manipulation of plasmid DNA and PCR products. Electrocompetent *E. coli* S17.1 λ*pir* cells were generally transformed with integrative suicide plasmids containing an RP4 origin of transfer determinant in order to facilitate plasmid transfer into methanotrophs by conjugation. *E. coli* transformants were typically selected on LB agar plates containing antibiotics at the following working concentrations: kanamycin (50 μg ml^{-1}); ampicillin (50-100 μg/ml^{-1}); gentamicin (15 μg ml^{-1}); tetracycline (12.5 μg ml^{-1}). All antibiotics used in this study were filter sterilised with a 0.22 μm filter and aseptically added to the cooled medium. All the bacterial strains and plasmids used in this study are shown in Table 2.1.

2.2 Cultivation and maintenance of bacterial strains

All solutions and growth media were prepared with Milli-Q water and sterilised by autoclaving at 15 psi for 15 minutes at 120 °C.

2.2.1 Bacterial strain purity check and microscopy

All bacterial strains were handled and cultivated axenically to minimise contamination. The purity of bacterial strains was routinely checked using light microscopy at 1000 x magnification under oil immersion. As well as microscopy, purity of stock methanotrophic cultures was checked by plating onto nutrient agar plates, which were incubated aerobically at 37 °C for at least two days. Identity of stock methanotrophs obtained from culture collections were confirmed by sequencing the 16S subunit of the ribosomal RNA and the sequence searched against sequences published in GenBank (http://www.ncbi.nlm.nih.gov/).

Table 2.1 Bacterial strains and plasmids used in this study. Abbreviations, *Mc*, *Methylococcus*; *Mm*, *Methylomonas*; *Ms*, *Methylosinus*; *Mb*, *Methylobacter*; *Mcys*, *Methylocystis*; *M*, *Methylocella*; Gm^R, gentamicin resistance; km^R, kanamycin resistance, tet^R, tetracycline resistance; Ap^R, ampicillin resistance.

Strains/Plasmids	Description	Reference/source
Strains		
Escherichia coli TOP10	F^-, ***mrcA***, *endA1*, *rec1*, ϕ80*lacZ*ΔM15, Δ*lacZX74*, *deoR*, *araD139*, *galK*, *rpsL* (Str^R), *nupG*, Δ(*mrr-hsdRMS-mcrBC*)	Invitrogen
Escherichia coli S17.1 λ*pir*	*recA1 thi pro hsdR*$^-$ RP4-2Tc::Mu Km::Tn*7* λ*pir*	(Simon *et al.*, 1983)
Mc. capsulatus Bath	Wild-type strain	Warwick culture collection
Mm. methanica S1	Wild-type strain	Warwick culture collection
Mm. rubra	Wild-type strain	Warwick culture collection
Ms. trichosporium OB3b	Wild-type strain	Warwick culture collection
Ms. sporium 5	Wild-type strain	Warwick culture collection
Mb. agile A20	Wild-type strain	Warwick culture collection
Mcys. parvus	Wild-type strain	Warwick culture collection
M. silvestris BL2	Wild-type strain	Warwick culture collection
Ms. sporium:Δ*mmoX*1	Δ*mmoX*1: Gm^R	This study
Ms. sporium:Δ*mmoX*2	Δ*mmoX*2: Gm^R	This study
Ms. sporium:Δ*mmoX*1:pVK104	Δ*mmoX*1 complemented with *Mc. capsulatus* (Bath) sMMO operon: Gm^R, Km^R	This study
Ms. sporium:Δ*mmoX*2:pVK100Sc	Δ*mmoX*1 complemented with *Ms. trichosporium* OB3b sMMO operon: Gm^R, Km^R	This study
Mc. capsulatus [pMHA011]	GFP reporter strain, Gm^R	This study
Mc. capsulatus [pMHA021]	XylE reporter strain, Gm^R	This study
Mc. capsulatus [pMHA034]	LacZ reporter strain, Gm^R	This study
Mc. capsulatus [pMHA035]	Km^R reporter strain, Gm^R	This study
Mc. capsulatus [pMHA036]	LacZ reporter strain, Gm^R	This study
Mc. capsulatus [pMHA201]	GFP reporter strain, Gm^R	This study

Mc. capsulatus:Δ*mmoR* [pMHA012]	GFP reporter strain, Gm^R, Km^R	This study
Mc. capsulatus:Δ*mmoG* [pMHA012]	GFP reporter strain, Gm^R, Km^R	This study
Mc.capsulatus:ΔMCA0705	ΔMCA0705 strain, Gm^R	This study
Mc.capsulatus:ΔMCA1883	ΔMCA1883 strain, Gm^R	This study
Mc.capsulatus:ΔMCA2590	ΔMCA2590 strain, Gm^R	This study
M. silvestris [pMGA203]	GFP reporter strain, Km^R	This study
Plasmids		
pUC19	Ap^R, cloning vector	(Vieira & Messing, 1982)
pK18mob	Km^R, RP4-mob, mobilizable cloning vector	(Schafer *et al.*, 1994)
pCR2.1-TOPO	Km^R, Ap^R, PCR product cloning vector	TA TOPO Cloning Kit (Invitrogen)
p34S-Gm	Source of Gm^R cassette	(Dennis & Zylstra, 1998)
pCM130	BHR *xylE* promoter probe vector, tet^R	(Marx & Lidstrom, 2001)
pCM132	BHR *lacZ* promoter probe vector, Km^R	(Marx & Lidstrom, 2001)
pMJ153	Source of *gfp* and *oriT*	D. A. Hodgson, University of Warwick
pDAH274	LacZ promoter probe vector	D. A. Hodgson, University of Warwick
pDAH350	Gm^R, cloning vector	D. A. Hodgson, University of Warwick
pBSL202	Mini-Tn*5* transposon vector, Gm^R	(Alexeyev *et al.*, 1995)
pAG408	Mini-Tn*5* transposon vector, containing *gfp*, Gm^R	(Suarez *et al.*, 1997)
pMHA048A	pUC19 containing 1670 bp *kpn*I fragment of pAG408	This study
pMHA048B	pUC19 containing 4498 bp *kpn*I fragment of pAG408	This study
pVK104	Km^R, BHR containing the *Ms. trichosporium* OB3b sMMO operon	(Martin & Murrell, 1995)
pVK100Sc	Km^R, BHR containing the *Mc. capsulatus* (Bath) sMMO operon	(Lloyd *et al.*, 1999b)
pMHA001	pDAH350 containing *oriT* from pMJ153, Gm^R	This study
pMHA010	pMHA001 containing *gfp* from pMJ153, Gm^R	This study
pMHA011	pMHA010 containing *mmoX* σ^{54} promoter from *Mc. capsulatus*, Gm^R	This study

pMHA012	pK18mob containing *mmoX* σ^{54} promoter and *gfp* from pMHA011, *Km*^{R}	This study
pMHA020	pMHA001 containing *xylE* from pCM130, *Gm*^{R}	This study
pMHA021	pMHA020 containing *mmoX* σ^{54} promoter from *Mc. capsulatus*, *Gm*^{R}	This study
pMHA034	pMHA011 containing *lacZ* amplified from pDAH274, but without *gfp*	This study
pMHA035	pMHA011 containing *Km*^{R} amplified from pDAH274, but without *gfp*	This study
pMHA036	pMHA034 containing a shorter version of the *mmoX* σ^{54} promoter from *Mc. capsulatus*, *Gm*^{R}	This study
pMHA199	pCM130 containing *Km*^{R} gene instead of *tet*^{R}	This study
pMHA200	pCM132 containing *gfp* from pMJ153 instead of *lacZ*	This study
pMHA201A	pCR2.1-TOPO containing pMMO σ^{70} promoter from *Mc. capsulatus*, *Km*^{R}	This study
pMHA201	pMHA200 containing pMMO σ^{70} promoter from pMHA200A	This study
pMHA202	pMHA201 containing N-pMMOB	This study
pMHA203A	pCR2.1-TOPO containing sMMO σ^{54} promoter from *M. silvestris*, *Km*^{R}	This study
pMHA203	pMHA200 containing sMMO σ^{54} promoter from pMHA203A	This study
pMHA204	pMHA200 containing sMMO σ^{54} promoter from *Mc. capsulatus*	This study
pMHA100	pDAH350 containing 5' region of *mmoX* σ^{54} promoter, pMMO σ^{70} promoter, *mmoX* and *oriT*, *Gm*^{R}	This study
pMHA500	Km^R, Gm^R, pK18mob containing *mmoG*::*mmoX*1::*Gm*^{R} cassette	This study
pMHA501	Km^R, Gm^R, pK18mob containing *mmoX*2::*Gm*^{R} cassette	This study
pMHA502	Km^R, Gm^R, pK18mob containing MCA0706::*Gm*^{R} cassette	This study
pMHA504	Km^R, Gm^R, pK18mob containing MCA1883::*Gm*^{R} cassette	This study
pMHA506	Km^R, Gm^R, pK18mob containing MCA2590::*Gm*^{R} cassette	This study

2.2.2 *Escherichia coli*

Escherichia coli strains were routinely cultivated on Luria-Bertani (LB) medium (Sambrook *et al.*, 1989). Luria-Bertani liquid cultures were incubated on a orbital shaker (200 rpm) at 37 °C. LB agar plates were prepared with the addition of 1.5 % (w/v) Bacto agar (Difco) prior to autoclaving. *E. coli* strains were stored at -80 °C for long-term storage in the presence of 15 % (v/v) sterile glycerol.

2.2.3 Methanotrophs

A Nitrate Mineral Salt (NMS) medium (Whittenbury *et al.*, 1970) was routinely used to culture methanotrophs. NMS agar plates were prepared with the addition of 1.5 % (w/v) Bacto (Difco) agar prior to autoclaving. The composition of the NMS medium was as follows:

Solution 1: Salt solution (10x stock)

KNO_3	10 g
$MgSO_4.7H_2O$	10 g
$CaCl_2.2H_2O$	2 g

Dissolved in 800 ml Milli-Q water and diluted to 1 litre.

Solution 2: Iron EDTA solution (1000x stock)

Fe-EDTA	0.38 g

Dissolved in 80 ml Milli-Q water and diluted to 100 ml (Concentration of stock Iron-EDTA solution = 1 mM).

Solution 3: Molybdate solution (1000x stock)

$NaMoO_4$	0.26 g

Dissolved in 800 ml Milli-Q water and diluted to 1 litre.

Solution 4: Trace elements solution (1000x stock)

$FeSO_4$	500 mg
$ZnSO_4.7H_2O$	400 mg
H_3BO_3	15 mg
$CoCl_2.6H_2O$	50 mg

Na-EDTA	250 mg
$MnCl_2.4H_2O$	20 mg
$NiCl_2.6H_2O$	10 mg

Dissolved in 800 ml Milli-Q water and diluted to 1 litre.

Solution 5: Copper solution (1000x stock)

$CuSO_4.5H_2O$	25 mg

Dissolved in 80 ml Milli-Q water and diluted to 100 ml (Concentration of stock copper solution = 1 mM).

Solution 6: Phosphate buffer solution (100x stock)

$Na_2HPO_4.12H_2O$	71.6 g
KH_2PO_4	26.0 g

Dissolved in 800 ml Milli-Q water. The pH was adjusted to 6.8 and diluted to 1 litre. Aliquots of the phosphate buffer was autoclaved separately.

Methanotrophs were typically cultivated in 1 x NMS medium, which was prepared by diluting 100 ml of the 10 x salt solution to 1 litre and adding 1 ml each of solutions 2 – 5. For solid medium, 15 g l^{-1} Bacto agar (Difco) was added prior to autoclaving. Sterile phosphate buffer (10 ml l^{-1}) was added to the cooled medium after autoclaving. Antibiotics were added as and when required at this stage at the following concentrations, kanamycin (15 μg ml^{-1}) and gentamicin (5 μg ml^{-1}). Low-copper medium were prepared for sMMO expression by omitting solution 5. For pMMO expression, Solution 5 was added to a final concentration ≥ 1 μM. Normal 1 x NMS medium contains 1 μM Fe-EDTA, however, for excess iron condition, up to 5 μM Fe-EDTA was used.

Methanotrophs were routinely cultivated in 250 ml conical flask containing 50 ml NMS medium. Flasks were sealed with suba-seals and gassed with 50 ml (i.e. ~20 %) methane/carbon dioxide (95 %/5 % (v/v) mix respectively). All methanotrophic cultures were incubated at 30 °C with shaking at 200 rpm, apart from *Mc. capsulatus*, which was incubated at 45 °C. A typical methanotroph culture took about 4-6 days to reach stationary phase (OD_{540} 0.8). Methanotrophs grown on NMS agar plates were incubated in a gas-tight container under a methane/air/carbon dioxide atmosphere (50/45/5 % (v/v) respectively) at the appropriate temperature. The gas was replenished

every two days until colonies formed, usually within 5-10 days depending on the strain.

Large scale cultivation of methanotrophs was carried out in 5 L fermentors (Inceltech LH Series 210) with a continuous flow of air (1 L min^{-1}) and methane (140 ml min^{-1}). The dissolved oxygen level in the fermentor vessel was maintained above 5 % by adjusting the agitation speed and air flow rate to prevent limitation of oxygen. pH of the culture was maintained between 6.8 – 7.2 with the automatic addition of 0.5 M HCl or 0.5 M NaOH. Growth was monitored by measuring the OD_{540} using a Beckman DU-70 spectrophotometer and also by monitoring the dissolved oxygen tension in the fermentor vessel using an oxygen electrode. Typically 4.5 L of NMS medium was inoculated with 6-8 x 50 ml overnight flasks cultures (i.e. 10 % (v/v) inoculum) grown to mid-late exponential phase (~OD_{540} 0.3-0.6). Cells were harvested during mid exponential phase (OD_{540} 4-6) and were used immediately for enzyme assays, or stored at -80 °C by resuspending the cells in minimum volume of 20 mM Tris-Cl (pH 7.0) and drop frozen in liquid nitrogen.

2.3 Extraction of nucleic acid

2.3.1 Genomic DNA extraction from methanotrophs

Genomic DNA was extracted from methanotrophs using methods described previously (Marmur, 1961). DNA from CsCl gradients were further purified by removing ethidium bromide and CsCl according to method of Sambrook *et al.*, (1989). On some occasions, the ultracentrifugation step was omitted and the DNA was isolated and purified from the cell lysed by multiple extractions with phenol, chloroform, isoamyl alcohol (25:24:1 (v/v) ratio) followed by ethanol precipitation.

2.3.2 Total RNA extraction from methanotrophs

Total RNA was isolated from methanotrophs using a hot-phenol method (Gilbert *et al.*, 2000) from 4 ml of exponential phase (OD_{540} 4-6) fermentor cultures or from 50 ml flask cultures (OD_{540} 0.3-0.6) expressing either pMMO or sMMO. All solutions used for the isolation of total RNA were prepared using diethylpyrocarbonate (DEPC) treated water with RNAse-free filter tips. The quality of the RNA was checked by running a small portion of the sample on a 1.2 % (w/v) TBE-agarose gel.

DNA was removed from the total RNA samples using 2-4 units of DNase (Promega) per μg of total RNA, according to the manufacturer's instructions. Removal of all traces of DNA was confirmed by the absence of a 16S rRNA PCR product.

2.3.3 Small-scale plasmid extraction from *E. coli* (mini-prep)

Small scale plasmid preparations were carried out using 1.5 ml overnight *E. coli* cultures using the method of Sambrook *et al.*, (1989). Plasmids required for direct sequencing were isolated and purified using the Qiaprep Miniprep Kit (Qiagen) according to the manufacturer's instructions.

2.3.4 Large-scale plasmid extraction from *E. coli* (maxi-prep)

Large scale plasmid preparations were carried out using the Qiaprep Maxiprep Kit (Qiagen) according to the manufacturer's instructions. For high-copy number plasmids, 100 ml *E. coli* cultures were used and 500 ml cultures were used for low-copy number plasmids.

2.4 Techniques for nucleic acid manipulation

2.4.1 Quantification of DNA/RNA

Concentrations of DNA and RNA were routinely estimated from agarose gel by comparison to a known amout of DNA in 1 kb ladder (Invitrogen) or by measuring the absorbance ratio at 260/280 nm using a Nano-drop spectrophotometer.

2.4.2 DNA restriction digests

All DNA restriction digestions were carried out with enzymes from MBI Fermentas according to the manufacturer's recommendations.

2.4.3 DNA purification

DNA fragments from enzymatic reactions such as restriction digestion were routinely excised from TBE or TAE agarose gel and the DNA purified using the QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer's instructions. PCR products were also routinely purified using this kit.

2.4.4 Dephosphorylation

DNA ends of plasmid DNA digested with single restriction enzyme were dephosphorylated with alkaline phosphatase from calf intestine (Roche) according to the manufacturer's instructions. All dephosphorylated DNA fragments were purified (Section 2.4.3) prior to ligation.

2.4.5 DNA ligations

T4 DNA ligase (Fermentas) was used to perform ligations with vector DNA and DNA insert and self-ligation of linear DNA according to the manufacturer's instructions. Typically, 60-400 ng of vector DNA was ligated with 60-1200 ng of insert DNA. For self-ligation, typically 500 ng genomic DNA digest was self-ligated in a 50 µl total volume.

2.4.6 Cloning PCR products

PCR products were cloned into pCR2.1-TOPO vector using the TOPO TA cloning Kit (Invitrogen) according to the manufacturer's instructions. Typically 2 µl of the TOPO cloning reaction was added to a one shot *E. coli* TOP10F chemically competent cells for transformation.

2.4.7 Agarose gel electrophoresis

DNA fragments were separated on 0.8 – 2 % (w/v) low-melting agarose in 1 x TBE buffer using Flowgen Minigel Systems. Ethidium bromide (0.5 µg ml^{-1}) was added prior to casting the gel and visualised on a UV transilluminator. DNA 1 kb ladder (Invitrogen or Fermentas) was used to estimate sizes of DNA fragments.

2.5 Bacterial genetic modification

2.5.1 Methanotroph conjugation

The transfer of plasmid containing RP4-*mob* from *E. coli* to methanotrophs was based on the method described previously (Martin & Murrell, 1995). A 10 ml overnight *E. coli* S17.1 λ*pir* culture, containing RP4-*mob* plasmid, was collected on a 0.2 μm pore-size nitrocellulose filter (Millipore). The *E. coli* donor strain was washed twice with 50 ml NMS. A 50 ml methanotroph culture grown to mid exponential phase (OD_{540} 0.2-0.5) was also collected on the same filter and washed again with 50 ml NMS medium. The filter was placed on an NMS agar plate containing 0.02 % (w/v) proteose peptone and incubated for 24 hours at 30 °C with methane except for *Mc. capsulatus*, which was incubated at 37 °C for 24 hours.

Following incubation, the cells were washed with 10 ml NMS and collected by centrifugation (7,000 x g for 10 min) before resuspending the cells in 1 ml NMS. Aliquots (50-100 μl) of the cells were spread onto NMS plates containing selective antibiotics and incubated at the appropriate temperature. Colonies typically formed on the plates after 8-12 days. (Note: The *E. coli* S17.1 λ*pir* strain has chromosomally integrated conjugal transfer functions, thus allowing transfer of plasmid to occur by means of a biparental mating without a helper plasmid).

2.5.2 Preparation and transformation of chemically competent *E. coli*

Chemically competent *E. coli* cells were prepared by $CaCl_2$ treatment as described in Sambrook *et al.*, (1989). The cells were resuspended in 2 ml of ice-cold 0.1 M $CaCl_2$ containing DMSO (140 μl per 4 ml resuspended cells) for each 50 ml of original culture and aliquots of 100 μl were frozen rapidly in liquid nitrogen and stored at -80 °C.

The chemically competent *E. coli* cells were transformed by adding plasmid DNA or ligation mix up to 10 % volume of the competent cells and incubating the cells on ice for 15-30 min. The cells were heat shocked by incubating the cells at 42 °C for 1 min and then placed on ice immediately for 2 min following the addition of SOC (0.5-1 ml) medium (Sambrook *et al.*, 1989). The cells were recovered at 37 °C shaking incubator (200 rpm) for 1 hour and aliquots (10-100 μl) of the respective transformants

were spread onto LB agar plates containing selective antibiotics and X-gal (40 μg ml^{-1}) when necessary and incubated at 37 °C for 18-24 hours.

2.5.3 Preparation and transformation of electrocompetent *E. coli*

A 500 ml exponentially growing *E. coli* S17.1 λ*pir* culture (OD_{600} 0.5) was chilled on ice for 15 min and then centrifuged at 4,000 x g for 15 min. The supernatant was decanted and the cell pellet was resuspended in 500 ml ice cold sterile water and centrifuged. Once again the supernatant was decanted and the cell pellet was resuspended in 250 ml sterile water and centrifuged. This time the cell pellet was resuspended in 10 ml ice cold sterile 10 % (v/v) glycerol and centrifuged. Finally the cell pellet was resuspended in 1 ml of ice cold sterile 10 % (v/v) glycerol. Aliquots (40 μl) of the resuspended cells were frozen in liquid nitrogen and stored at -80 °C.

The electrocompetent *E. coli* S17.1 λ*pir* cells were transformed by adding up to 2 μl of plasmid DNA or ligation mix and incubating on ice for 1 min. The cells and DNA mix was transferred to a 0.2 cm electroporation cuvette and using a Bio-Rad GenePulser, an electric field of 2.5 kV cm^{-1} at 25 μF and 200 Ω was applied. Following the electric pulse, the cells were immediately suspended in 1 ml SOC medium and allowed to recover with shaking (200 rpm) at 37 °C for 1 hour. Aliquots (50-100 μl) of the respective transformants were spread onto LB agar plates containing selective antibiotics.

2.6 Polymerase chain reaction (PCR)

PCR amplifications were performed in 50 μl total volume of reaction mixture using a Hybaid Touchdown Thermal Cycling System. *Taq* DNA polymerase and dNTPs were purchased from Fermentas and custom primers were obtained from Invitrogen. The composition of a typical PCR reaction is shown in Table 2.2.

Table 2.2. Reagent composition in a typical PCR reaction. [1]DMSO/Betaine was made by mixing 2.6 M Betaine with 2.6 % (v/v) DMSO to give a 2X stock solution. DMSO/Betaine was only added to difficult PCR reactions in order to increase the specificity and efficiency of amplification when amplifying DNA containing high GC content.

Reagents	[Stock]	[Final]	Volume (µl)
PCR buffer	10X	1X	5
dNTP mix	2.5 mM (each)	0.25 mM	5
$MgCl_2$	25 mM	2.5 mM	5
Primer (Forward)	10 µM	0.2 µM	1
Primer (Reverse)	10 µM	0.2 µM	1
DMSO/Betaine (Optional)[1]	2X	1X	25
Sterile deionised water	-	-	to 44 µl

To the reaction mix, 1 µl of purified genomic DNA or plasmid DNA containing 10-100 ng DNA was added. Diluted *Taq* DNA polymerase (i.e. 5 µl of 0.5u μl^{-1} *Taq* DNA polymerase) was always added after a 5 min hot-start at 94 °C to give a 50 µl total reaction volume. A typical program for a PCR reaction is shown in Table 2.3.

Table 2.3. A typical PCR programme. [1]The annealing temperature was varied from 50-60 °C and was usually 5 °C below the average melting temperature of the primers. [2]The extension time was also varied depending on the length of the PCR product. As a rule of thumb, a 1 min extension was used per kb of DNA amplified.

Step	Temp (°C)	Time (min)	Cycles
Hot-start	94	5	1
Add 2.5 units of *Taq* DNA polymerase			
Denaturation	94	1	30
Annealing	Variable[1]	1	
Extension	72	Variable[2]	
Final extenstion	72	10	1
Hold at 20 °C			

PCR reactions were routinely optimised by altering the template and primer concentrations and by varying the annealing temperature and extension time. The number of cycles was also increased for PCR reactions yielding low amounts of products.

2.6.1 Inverse PCR

Inverse PCR was used to amplify and sequence upstream and downstream DNA sequence from a known region of DNA sequence. Briefly, 1 μg of genomic DNA was digested with restriction enzymes that cut frequently. DNA was then self-ligated (Section 2.4.5) in 50 μl reaction volumes using T4 ligase (MBI Fermentas) to generate small circles. The ligation mix was then used directly as PCR template with outward facing primers designed near the end of the known DNA sequence. The PCR products were either cloned into pCR2.1-TOPO vector using the TA TOPO cloning kit (Section 2.4.6) or purified and sequenced directly using outward facing primers.

2.6.2 Error-prone PCR

GeneMorph II Random Mutagenesis Kit (Stratagene) was used to carry out error-prone PCR according to the manufacturer's instructions. The mutation frequency per kilobase of DNA was controlled by adjusting the initial target DNA amounts in the amplification reactions. Due to the low copy number of genomic DNA targets, the target DNA was initially PCR amplified from genomic DNA using normal *Taq* DNA polymerase and cloned into pCR2.1-TOPO vector (Section 2.4.6). The DNA target in the plasmid was subjected to random mutagenesis. For low frequency mutation (0-4.5 mutations kb^{-1}), it was recommended to use 500-1000 ng target DNA and for high frequency of mutation (9-16 mutations kb^{-1}) it was recommended to use 0.1-100 ng of target DNA (Note: The initial amount of target DNA required to achieve a particular mutation frequency refers to the amount of DNA to amplify and not the total amount of plasmid DNA template to be added to the reaction).

An example calculation of the template amount (ng) required for a high frequency mutation of a 505 bp DNA fragment in a 7863 bp plasmid.

$$\frac{\textbf{Target Amount (ng) x Template Length (bp)}}{\textbf{Target Length (bp)}} = \textbf{Template Amount (ng)}$$

$$\frac{\textbf{10 ng x 7863 bp}}{\textbf{505 bp}} = \textbf{156 ng}$$

2.6.3 Reverse transcriptase PCR (RT-PCR)

RT-PCR was performed with SuperScript II Reverse Transcriptase (Invitrogen). DNase-treated total RNA (0.5-1 μg) was added to 50 pmol gene-specific reverse primer and 1 μl dNTP mix (10 mM each) in a final volume of 12 μl. The mixture was heated to 65 ^{0}C for 5 min and then chilled on ice. To the reaction mixture, 4 μl of 5X first-strand buffer, 2 μl 0.1 M DTT and 1 μl (200 units) of SuperScript II RT were added to give a 20 μl final volume before incubation at 42 ^{0}C for 50 min, followed by a 15 min incubation at 70 ^{0}C to inactivate the RT enzyme. The cDNA (1-5 μl) was used as a template for PCR amplification, as described in Section 2.6, without further purification.

cDNA synthesis of low-abundunce mRNA was achieved using SuperScript III One-Step RT-PCR System with Platinum *Taq* DNA Polymerase (Invitrogen) according to the manufacturer's instructions.

2.7 DNA/RNA blotting, hybridisation and detection

2.7.1 Southern transfer of DNA to membranes

Approximately 3-5 μg aliquots of genomic DNA were digested with various restriction enzymes as described in Section 2.4.2. The DNA fragments were resolved on a 0.9 % (w/v) agarose gel in Tris-Acetate-EDTA (TAE) buffer using a Horizontal Gel System unit for 18-20 hours at 1.5 V cm^{-1}. Ethidium bromide was added directly to the gel prior to casting at a final concentration of 0.5 μg ml^{-1}. The DNA on the gel was visualised and photographed alongside a ruler so that the distances and sizes of the fragments from the molecular mass ladder could be used to correlate the sizes of the DNA fragments hybridised on the Southern blot. The DNA was denatured by submerging the gel for 30 min in Denaturation buffer (0.5 M NaOH, 1.5 NaCl) and transferred onto Hybond N+ membrane (Amersham Biosciences) by capillary blotting following the alkali transfer protocol provided by the manufacturer. The DNA on the membrane was fixed by UV cross linking for 30 seconds with a Stratalinker (Stratagene).

2.7.2 Construction of clone libraries and colony blots

Partial clone libraries were constructed by cloning size fractionated genomic DNA, separated and purified using methods described earlier, into the multiple cloning sites (MCS) of pUC18 or pUC19 vector. Transformants containing the recombinant DNA were selected on LB agar plates containing ampicillin (100 μg ml^{-1}) and X-gal. Insertion of a genomic DNA fragment into the MCS of pUC18 or pUC19 vector resulted in a white clone due to insertional inactivation of the *lacZα* gene, which encodes part of β-galactosidase.

Colony blots containing *E. coli* clones were prepared initially by picking only white colonies into 150 μl L-broth in 96 well plates and grown for 18-24 hours at 37 °C. The clones from the 96 well plates were streaked onto Hybond N+ membrane placed on the surface of LB agar containing selective antibiotics and incubated for 18-24 hours at 37 °C. The cells were lysed on the membrane by placing the membrane on 2 sheets of 3MM Whatman paper soaked in the following solutions in this order:

- 10 % (w/v) SDS for 5 min
- Denaturation buffer (0.5 M NaOH, 1.5 M NaCl) for 5 min
- Neutralising buffer (1 M Tris-HCl (pH 7.4), 1.5 M NaCl) for 5 min. This step was repeated twice.
- 2 x SSC (3 M NaCl, 0.3 M trisodium citrate, pH 7) for 5 min

The membrane was dried for 10 min at room temperature and the DNA was then fixed to the membrane by UV cross linking using a Stratalinker (Stratagene).

2.7.3 Blotting of RNA samples to membranes

The DNA-free RNA samples were prepared by adding 30 μl of RNA denaturation solution (600 μl formamide, 210 μl formaldehyde (37 % (v/v)), and 130 μl 10x MOPS buffer (pH 7.0)) to each sample and making the volume to 80 μl with DEPC-treated water and incubated at 65 °C for 5 min (Note: MOPS buffer was made from 0.2 M MOPS (pH 7.0), 20 mM sodium acetate and 10 mM EDTA). Then an equal volume of 20x SSC was added to each sample and stored on ice.

The blotting manifold was prepared by initially cleaning with 0.1 M NaOH and rinsing thoroughly with sterile water. Two sheets of 3MM Whatman paper was soaked with 20x SSC and placed on top of the vacuum unit of the apparatus and the wet Hybond N+ membrane (Amersham Biosciences), previously soaked in 20x SSC for 1 hour was placed on the bottom of the sample wells. The manifold was clamped together evenly and connected to the vacuum. The slots were filled twice with 10x SSC and gentle suction was applied until the fluid past through the membrane. The RNA samples were then loaded into the slots and was also allowed to pass through with gently suction. When all the samples passed through the membrane, each slot was washed twice with 1 ml of 10x SSC and the suction was applied for an additional 5 min to dry the membrane. The RNA was fixed to the membrane by UV cross linking.

2.7.4 Random labelling of DNA probes and DNA hybridisation

All DNA probes, PCR amplified or digested from plasmids, were purified using methods described in Section 2.4.3 prior to random labelling. DNA probes (~25 ng) were denatured by heating for 10 min at 100 °C using a thermocycler and then radiolabelled with 50 μCi [α-^{32}P] dGTP, 10 units of Klenow fragment, 3 μl dNTP mix (0.5 mM) and 2 μl of 10x Hexanucleotide mix (Roche) according to the manufacturer's instructions. The reaction was stopped with the addition of 2 μl of 0.2 M EDTA (pH 8.0) and the probe was denatured by the addition of NaOH to a final concentration of 0.4 M.

Prior to hybridisation of the labelled probe to the Southern, colony, or RNA blots, the membranes were prehybridised at 50 °C for 30 min in 1 ml hybridisation buffer (0.5 M sodium phosphate buffer (pH 7.2), 5 mM ethylenediamine tetra-acetate (pH 8.0), 7 % (w/v) sodium dodecyl sulphate) per cm^2 of Hybond N+ membrane. The membranes were hybridised with fresh hybridisation buffer at 50 °C for 16-24 hours.

2.7.5 Washing of immobilised nucleic acid and autoradiography

Membranes hybridised with radiolabelled probes were washed with 2x SSC containing 0.1 % (w/v) SDS. To remove background hybridisation, the stringency of the washes was increased by increasing the temperature and reducing the salt concentration. The hybridised blots were exposed to X-ray films (Fujifilm) placed in an autoradiography cassette with intensifying screens and placed in a -80 °C freezer for

varying times (1-72 hours) depending on the signal intensity. X-ray films were developed and fixed in accordance to the manufacturer's instructions. The hybridisation signals from the hybridised blots were also detected by exposing a phosphorimaging plate (Fujifilm) for 5-60 min at room temperature. The signal from the phosphorimaging plate was scanned using a FLA-5000 phosphorimager according to the manufacturer's instructions.

2.8 DNA sequencing and analysis

DNA sequencing reactions were performed by the Molecular Biology Service at the University of Warwick using a Dye Terminating Kit (PE Applied Biosciences) and the DNA signals were analysed using a 373A automated sequencing system. The DNA sequences were annotated using Chromas (Version 1.45) and Lasergene sequence analysis software tools.

2.9 Biochemical analysis

2.9.1 Preparation of cell-free extracts from bacterial strains

Cells were harvested during exponential growth phase, either from a 5 L fermentor culture (OD_{540} = 4-6) or from small batch cultures grown in 250 ml flasks (OD_{540} = 0.4-0.6), by centrifugation (10,000 x g, 15 min at 4 °C). The cell pellets were resuspended in a minimum volume of 25 mM MOPS buffer (pH 7) containing 1 mM benzamidine, 5 mM dithiothreitol and a few crystals of deoxyribonuclease. The cells were broken by two passages through a pre-cooled French Press (American Instrument Company) at 110 MPa. The cell-free extracts were then separated into soluble and particulate fractions by centrifugation (38,000 x g, 60 min at 4 °C). The cell-free extracts were used directly for enzyme assays or were rapidly frozen in liquid nitrogen and stored at -80 °C.

2.9.2 Protein quantification

Protein content in sample preparations was quantified using Bio-Rad reagent according to the manufacturer's instructions. Solutions of bovine serum albumin (0-100 μg) were used as standard. Each sample was measured at least three times and an average protein concentration was used for calculating specific enzyme activity.

2.9.3 SDS-PAGE analysis

Protein samples were separated by SDS-PAGE using an X-cell II Mini-Cell apparatus (Novex). A 4 % (w/v) stacking gel and a 12.5 % (w/v) resolving gel was typically used and prepared as follows:

4 % Stacking gel

• 40 % (w/v) Acrylamide	0.53 ml
• 2 % (w/v) Bisacrylamide	0.3 ml
• Stacking buffer (0.5 M Tris-HCl, pH 6.8)	1.36 ml
• Water	2.8 ml
• 10 % (w/v) SDS	50 µl
• 10 % (v/v) Glycerol	50 µl
• 10 % (w/v) Ammonium persulphate	0.5 ml
• TEMED	5 µl

12.5 % Resolving gel

• 40 % (w/v) Acrylamide	3.15 ml
• 2 % (w/v) Bisacrylamide	1.69 ml
• Resolving buffer (3 M Tris-HCl, pH 8.8)	2.5 ml
• Water	2.65 ml
• 10 % (w/v) SDS	100 µl
• 10 % (v/v) Glycerol	100 µl
• 10 % (w/v) Ammonium persulphate	0.5 ml
• TEMED	5 µl

Sample loading buffer

• Stacking buffer	250 µl
• 100 % (v/v) Glycerol	200 µl
• 0.5 % (w/v) Bromophenol blue	80 µl
• β-mercaptoethanol	100 µl
• 10 % (w/v) SDS	1.2 ml

5x Running buffer

- Glycine $72\ gl^{-1}$
- Tris base $15\ gl^{-1}$
- SDS $5\ gl^{-1}$
- Water to 1 litre

The protein samples were mixed with equivalent volume of sample loading buffer and boiled for 5 min before separating on a 12.5 % SDS-PAGE gel. Proteins were stained with Coomassie brilliant blue staining solution (0.1 % (w/v) Coomassie Brilliant blue R-250 dissolved in 40 % methanol, 10 % acetic acid and 50 % water) and destained in 40 % (v/v) methanol and 10 % (v/v/) acetic acid. Dalton Mark VII-L (Sigma) molecular mass marker was used to estimate molecular masses.

2.9.4 MS/MS analysis of polypeptides

Polypeptides of interest, from SDS-PAGE, were analysed by the Biological Mass Spectrometry and Proteomics Group at Warwick, using an in-line liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS). The MS/MS data were processed and searched against the appropriate databases using the Micromass Global Server 2.0 search engine.

2.10 Enzyme Assays

2.10.1 Naphthalene assay for sMMO activity

A naphthalene oxidation assay was routinely used for the qualitative detection of sMMO activity on NMS agar plates or in liquid cultures using methods described previously (Brusseau *et al.*, 1990; Graham *et al.*, 1992). sMMO is capable of oxidising naphthalene to naphthol, which can react with a zinc complex (tetrazotized *o*-dianisidine) to form a deep purple colour, thus indicating sMMO expression and activity.

2.10.2 Green Fluorescent Protein (GFP) assay

The GFP fluorescence was measured using a fluorimeter at an excitation wavelength of 475 nm and an emission wavelength of 510 nm (Note: The upper emission limit was 485 nm and the lower emission limit was 550 nm, which were integrated at a scan rate of 0.95 nm second^{-1} with one repetition). As and when appropriate, samples with high GFP activity were diluted in deionised water accordingly to prevent saturation of the detector. The fluorescence intensity from samples was represented as arbitrary units.

All GFP fluorescence was measured from cell-free extracts whose protein content was quantified using the Bio-Rad reagent (Section 2.9.2). The data obtained were normalised by expressing the arbitrary fluorescent units as a specific activity in units mg^{-1} protein. The mean specific activity, from at least two independent assays, is indicated in the results.

2.10.3 Catachol-2, 3 dioxygenase (XylE) assay

Catachol-2, 3 dioxygenase activity was measured using methods described previously (Kataeva & Golovleva, 1990; Zukowski *et al.*, 1983), with slight modifications. The qualitative detection and thus the functional expression of XylE was examined by either spraying or pipetting a solution of 0.1 M catechol (Sigma) directly onto colonies or liquid cultures. Catachol-2, 3 dioxygenase rapidly converts the colourless catechol to an intensively yellow oxidative product, 2-hydroxymuconic semialdehyde (Figure 2.1), thus colonies of cells or liquid cultures that express XylE become yellow/orange.

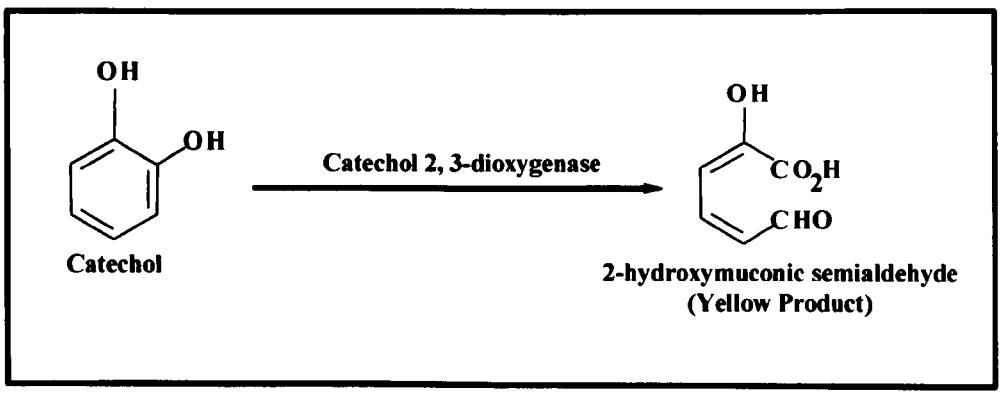


Figure 2.1 Enzymatic conversion of catechol to the yellow/orange product, 2-hydroxymuconic semialdehyde by catechol 2, 3-dioxygenase.

Quantitative assay for catechol 2, 3-dioxygenase was conducted in a 1 cm cuvette at 30 °C in a total volume of 1 ml containing the following: 0.88 ml of 50 mM Tris buffer (pH 7.5); 0.02 ml of 50 mM catechol dissolved in the Tris buffer; and 0.1 ml of cell-free extract or appropriate dilutions. The spectrophotometer was calibrated and blanked at 375 nm with the sample mixture before the addition of the cell free extracts. Catechol 2, 3-dioxygenase activity was calculated as the rate of change in optical density at 375 nm min^{-1} mg^{-1} of protein and finally expressed as nmoles of 2-hydroxymuconic semialdehyde formed mg^{-1} of protein min^{-1} (Note: A change in optical density of 1 min^{-1} is equal to 1 unit and one milliunit corresponds to the formation at 30 °C of 1 nmole of 2-hydroxymuconic semialdehyde min^{-1}). An average change of OD_{375} min^{-1} over 3 min was used to calculate specific activities.

An example calculation:

For an enzyme reaction with an average ΔOD_{375} of 0.1090 units min^{-1} and with a protein concentration of 37.65 mg ml^{-1}, the specific activity would be worked out as follows:

$$\textbf{0.109 units min}^{-1} = \textbf{109 milliunits min}^{-1}$$

The dilution factor needs to be taken into account. If the sample was diluted 50 fold and 100 µl was used in the enzyme reaction, the specific activity would be:

$$\frac{\textbf{109 milliunits min}^{-1} \textbf{ x 50}}{\textbf{3.765 mg}} = \textbf{1448 milliunits min}^{-1}\ \textbf{mg}^{-1}$$

Therefore, 1448 nmoles of 2-hydroxymuconic semialdehyde is formed per min per mg of protein

2.10.4 β-galactosidase assay

There are a number of chromogenic and fluorogenic substrates for detecting β-galactosidse activity (Sambrook *et al.*, 1989) and in this study a number of these substrates were used (Figure 2.2)

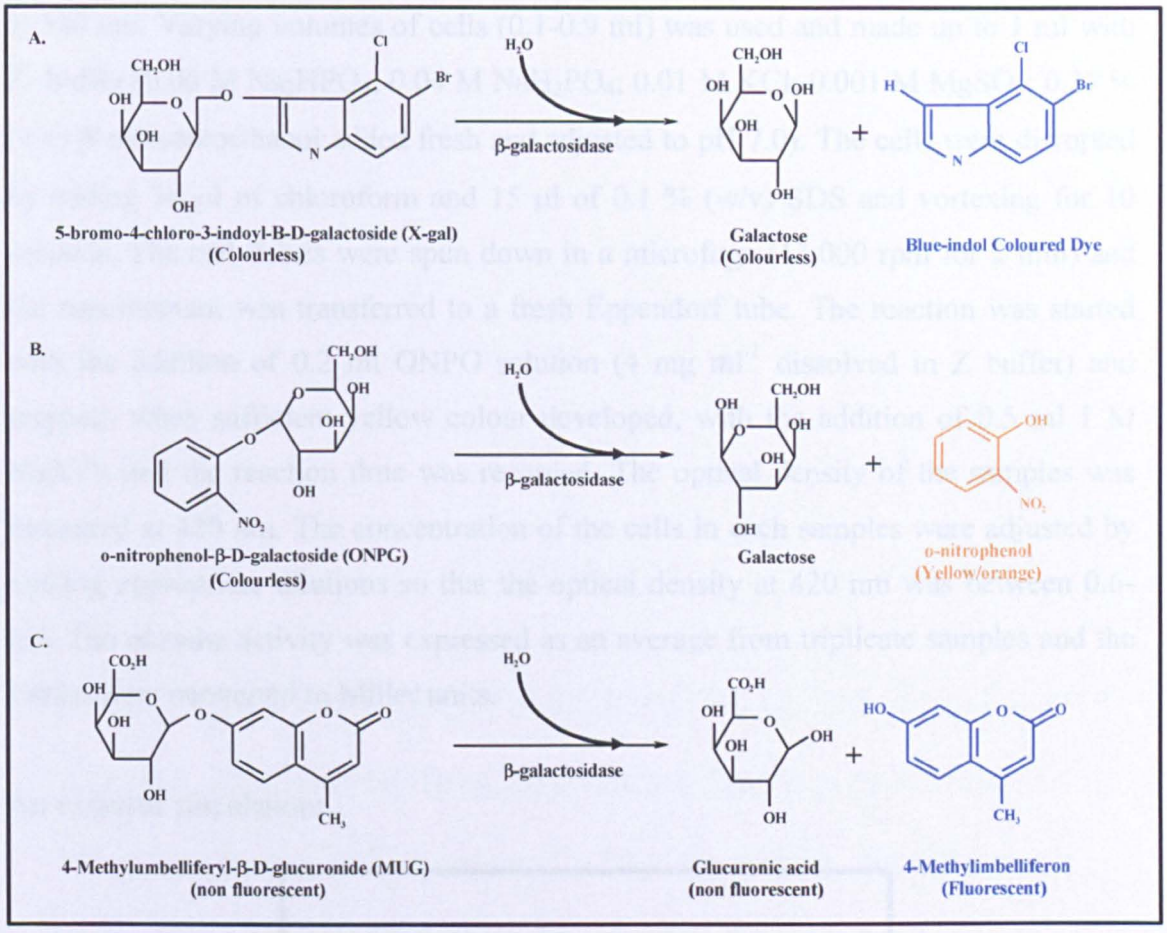


Figure 2.2 Various hydrolysis reactions of β-galactosidase using chromogenic substrates (A and B) and a fluorogenic substrate (C).

The *lacZ* gene encodes for β-galactosidase (LacZ) enzyme. The qualitative detection and thus the functional expression of *lacZ* genes was examined with the addition of X-gal, dissolved in DMSO to a final concentration of 40 μg ml^{-1}, either to the cooled medium or added directly onto the surface of the agar. The formation of blue colonies indicated the expression of *lacZ*. The MUG substrate was used as an alternative substrate for detecting β-galactosidase activity. MUG solution (4 mg ml^{-1} in DMSO) was pipetted onto the colonies and the plates were incubated at room temperature for 20 min. The colonies were then viewed under ultraviolet light for fluorescence.

β-galactosidase activity was measured quantitatively by following the conversion of *o*-nitrophenol-β-D-galactoside to o-nitrophenol using methods described previously (Miller, 1972). The assay was done on whole cells in 2 ml Eppendorf tubes in a final volume of 1 ml. The cell density was recorded by measuring the absorbance

at 540 nm. Varying volumes of cells (0.1-0.9 ml) was used and made up to 1 ml with Z- buffer (0.06 M Na_2HPO_4; 0.04 M NaH_2PO_4; 0.01 M KCl; 0.001 M $MgSO_4$; 0.27 % (v/v) β-mercaptoethanol added fresh and adjusted to pH 7.0). The cells were disrupted by adding 30 µl of chloroform and 15 µl of 0.1 % (w/v) SDS and vortexing for 10 seconds. The cell debris were spun down in a microfuge (13,000 rpm for 2 min) and the supernantant was transferred to a fresh Eppendorf tube. The reaction was started with the addition of 0.2 ml ONPG solution (4 mg ml^{-1} dissolved in Z buffer) and stopped, when sufficient yellow colour developed, with the addition of 0.5 ml 1 M Na_2CO_3 and the reaction time was recorded. The optical density of the samples was measured at 420 nm. The concentration of the cells in each samples were adjusted by making appropriate dilutions so that the optical density at 420 nm was between 0.6-0.9. The enzyme activity was expressed as an average from triplicate samples and the values were converted to Miller units.

An example calculation:

$$\frac{1000 \times OD_{420}}{t \times v \times OD_{540}} = \text{Miller Units}$$

t = time of the reaction in minutes

v = volume of culture used in the assay, in ml

For an enzyme reaction with an average OD_{420} of 0.9 after 10 minutes, using 0.5 ml culture with an OD_{540} of 0.3, the Miller Units for β-galactosidase would be:

$$\frac{1000 \times 0.9}{(10 \times 0.5 \times 0.3)} = 600 \text{ units}$$

Note: 1 Miller unit of β-galactosidase is defined as the amount of enzyme which produces 1 µmole of o-nitrophenol min^{-1} at pH 7.0.

Chapter 3

Screening of Obligate Methanotrophs for Regulatory Genes and Identification and Characterisation of Duplicate *mmoX* Genes in *Ms. sporium* 5: Cloning, Sequencing and Mutational Analysis

3.1 Introduction

Bacteria are highly versatile organisms that can readily adapt to their environment. They control their gene expression in response to environmental stimuli so that at any given time they synthesise the gene products that are essential for survival in that environment. The amounts of a particular enzyme or structural protein synthesised are determined by a number of factors, including the cell's nutritional status. Although the mechanism by which the concentration of a specific gene product is regulated varies widely, global gene regulation in bacteria is predominantly controlled at the level of transcription. Proteins that are involved in essential cellular processes, such as transcription and translation are generally expressed constitutively. The genes that encode these types of proteins are called 'housekeeping genes' and their level of transcription is not generally regulated appreciably during exponential growth phase. However, in bacteria many gene products are required only occasionally and they are regulated by an on-off switch and thus enable synthesis of these gene products as and when required. In most circumstances, when the transcription is in the off state, a basal level of transcription remains, however, there are a few cases when transcription is completely switched off (Freifelder, 1987).

sMMO-containing methanotrophs such as *Mc. capsulatus* and *Ms. trichosporium* have the particular metabolic flexibility in that they can readily adapt to growth under copper depleted conditions. However, until relatively recently, very little was known about the molecular regulation of the expression of MMO by copper ions. In previous studies, it has been shown that the transcription of the sMMO operon is initiated from a σ^{54}-dependent promoter that is tightly regulated by copper at the level of transcription (Csaki *et al.*, 2003; Nielsen *et al.*, 1996). Transcription from the sMMO σ^{54} promoter is active only under low copper-to-biomass ratio (<0.25 μM $CuSO_4$) and is totally repressed under high copper-to-biomass ratio (>0.25 μM $CuSO_4$). The strict transcriptional regulation of σ^{54} promoters, in response to various environmental signals, is a characteristic feature of these promoters (Shingler, 1996).

The identification and characterisation of the regulatory genes *mmoR* and *mmoG* in *Mc. capsulatus* and *Ms. trichosporium* (Csaki *et al.*, 2003; Stafford *et al.*, 2003) has given further insights into the molecular regulation of sMMO expression. MmoR is a σ^{54}-dependent transcriptional activator, which is another common feature of transcription mediated from a σ^{54}-dependent promoter. The function of MmoG,

which has significant identity to the large subunit of the bacterial chaperonin, GroEL, is unknown, however, it has been shown through construction of knock-out mutants by marker-exchange mutagenesis that MmoR and MmoG are essential for sMMO expression. The role of the σ^{54}-dependent transcriptional activator, MmoR, as the name suggests would be involved in transcriptional initiation. This role is now established, as is shown by results using transcriptional fusions between the sMMO σ^{54} promoter and GFP (Csaki *et al.*, 2003) and also in this study by using integrative suicide plasmids (Chapter 5). No GFP activity was detected in the *Mc. capsulatus* Δ*mmoR* mutant strains containing these plasmids under sMMO expressing conditions, thus indicating its crucial role in transcription initiation. Since there are no reports of bacterial chaperonins functioning in gene regulation, it was suggested from the initial mutational analysis that MmoG may play a role in sMMO assembly (Stafford *et al.*, 2003). Interestingly, similar experiments using promoter probe plasmids in *Mc. capsulatus* Δ*mmoG* strains indicated that MmoG was also crucial for transcription (Chapter 5). The gene encoding the sigma factor, σ^{54} (*rpoN*), in *Ms. trichosporium* has been cloned and sequenced. Knock-out mutants of *rpoN* abolished sMMO expression and the ability of *Ms. trichosporium* to fix nitrogen. This suggested that σ^{54} is also required for the initiation of transcription of the genes responsible for N_2 fixation (Stafford *et al.*, 2003). Several attempts to mutate *rpoN* in *Mc. capsulatus* were made without success (Csaki *et al.*, 2003). These data suggest that RpoN is a universal sigma factor responsible for transcriptional initiation from many σ^{54} promoters, whereas the σ^{54}-dependent activator, MmoR, is operon specific and is crucial for the transcriptional regulation of the sMMO operon.

In *Mc. capsulatus*, two additional genes, *mmoS* and *mmoQ*, were identified, which showed significant identify to two-component sensor-regulator system (Csaki *et al.*, 2003). Two-component systems are widespread in bacteria and are generally involved in stimulus-response coupling mechanisms in sensory systems that detect environmental changes. They have become well adapted to modulate a wide variety of cellular activities and thus mediate specific responses (Stock *et al.*, 2000). In the *Mc. capsulatus* genome, numerous putative two-component systems have been identified on the basis of sequence similarities, although their functions are largely unknown. Although the exact function of MmoQ and MmoS is not known, it was hypothesised that they may be involved in the copper-dependent regulation of sMMO transcription

(Figure 1.11) (Csaki *et al.*, 2003). The MmoS protein is similar to a typical sensor protein domain containing histidine kinase and is thought to sense copper ions (via an unknown mechanism). MmoQ is assumed to regulate transcription initiation through the interaction with MmoR. At the beginning of this study, these genes had not been identified in the sMMO gene clusters from other methanotrophs and therefore it was not clear how widespread their involvement in regulation of sMMO expression was or whether their occurrence was specific to methanotrophs that could produce sMMO.

In light of these data, it was necessary to further identify and characterise these regulatory genes in other obligate methanotrophs and investigate whether *mmoR*, *mmoG*, *mmoQ* and *mmoS* are specific to only sMMO expressing methanotrophs. This formed the basis of the initial study, which led to the cloning and sequencing of the genes encoding the sMMO enzyme in *Ms. sporium* strain 5 and the identification of duplicate *mmoX* genes. One of the most surprising results detailed below revealed that the sMMO genes of *Ms. sporium* strain 5 are duplicated, a phenomenon that had not previously been observed with sMMO genes in methanotrophs. Despite the initial characterisation of sMMO from *Ms. sporium* at the biochemical level (Pilkington & Dalton, 1991), very little was known at the molecular level about the copper-dependent expression of MMO in *Ms. sporium*. In this study, the duplication of *mmoX* was thoroughly investigated by means of transcriptional, biochemical, and mutational analysis. In addition, RNA dot blotting techniques were applied to gain key information on the copper-dependent transcriptional regulation of MMO in *Ms. sporium*.

3.2 Screening of obligate methanotrophs for sMMO regulatory genes

To identify homologues of the regulatory genes, *mmoR*, *mmoG*, *mmoQ*, and *mmoS* in various obligate methanotrophs, a Southern hybridization approach was taken using ^{32}P-labelled gene probes, PCR amplified from either *Mc. capsulatus* or *Ms. trichosporium*. Genomic DNA from a number of type I and type II methanotrophs were digested with various restriction enzymes (Figure 3.1). Initially *mmoR*, taken from pTJS172 (Smith *et al.*, 2002), was used as a probe for Southern hybridisation. Other than *Ms. trichosporium* OB3b, the only other strain that gave signals for *mmoR* was *Ms. sporium* (Figure 3.2). Radioactive signals for the presence of *mmoR* homologues in *Ms. sporium* could be seen with all the digests of chromosomal DNA

from that organism. The 2.3 kb *Eco*RI fragment was the largest fragment that hybridised with the *mmoR* probe and therefore was targeted for cloning by constructing a size fractionated clone library with pUC19 cloning vector in *E. coli* TOPO10F. A total of 624 clones were screened by colony hybridisation using *mmoR* again as a gene probe (Figure 3.3). Plasmid DNA of pTJS172 containing *mmoR* from *Ms. trichosporium*, was used as a positive control. Two clones (clones 343 and 407) were identified and designated as pHA001 and pHA002, respectively. Both clones were fully sequenced by primer walking.

Separate Southern blots containing digested chromosomal DNA from various methanotrophs were also probed individually with *mmoG*, *mmoQ*, and *mmoS*. The *mmoG* probe was PCR amplified from *Ms. trichosporium* using primers *mmoG*F_214 (5'-ACC AAG GAT GGC GTG ACC G-3') and *mmoG*R_1288 (5'-TGC GTT GCA TGC GTT CCT TG-3') yielding a 1075 bp fragment. The *mmoQ* and *mmoS* probes were PCR amplified from *Mc. capsulatus* using primers *mmoQ*F_1430 (5'-TCA CCG GCC TGC CCA ACC-3'), *mmoQ*R_1882 (5'-CGT TAC GCC CGT TCT GCT TGA C-3'), *mmoS*F_160 (5'-GCG CTG GAT GGA CTG CTG CTC-3'), and *mmoS*R_1175 (5'-TTC CCA TGC TCC GCC TTG AG-3') yielding a 453 bp and a 1016 bp fragment, respectively.

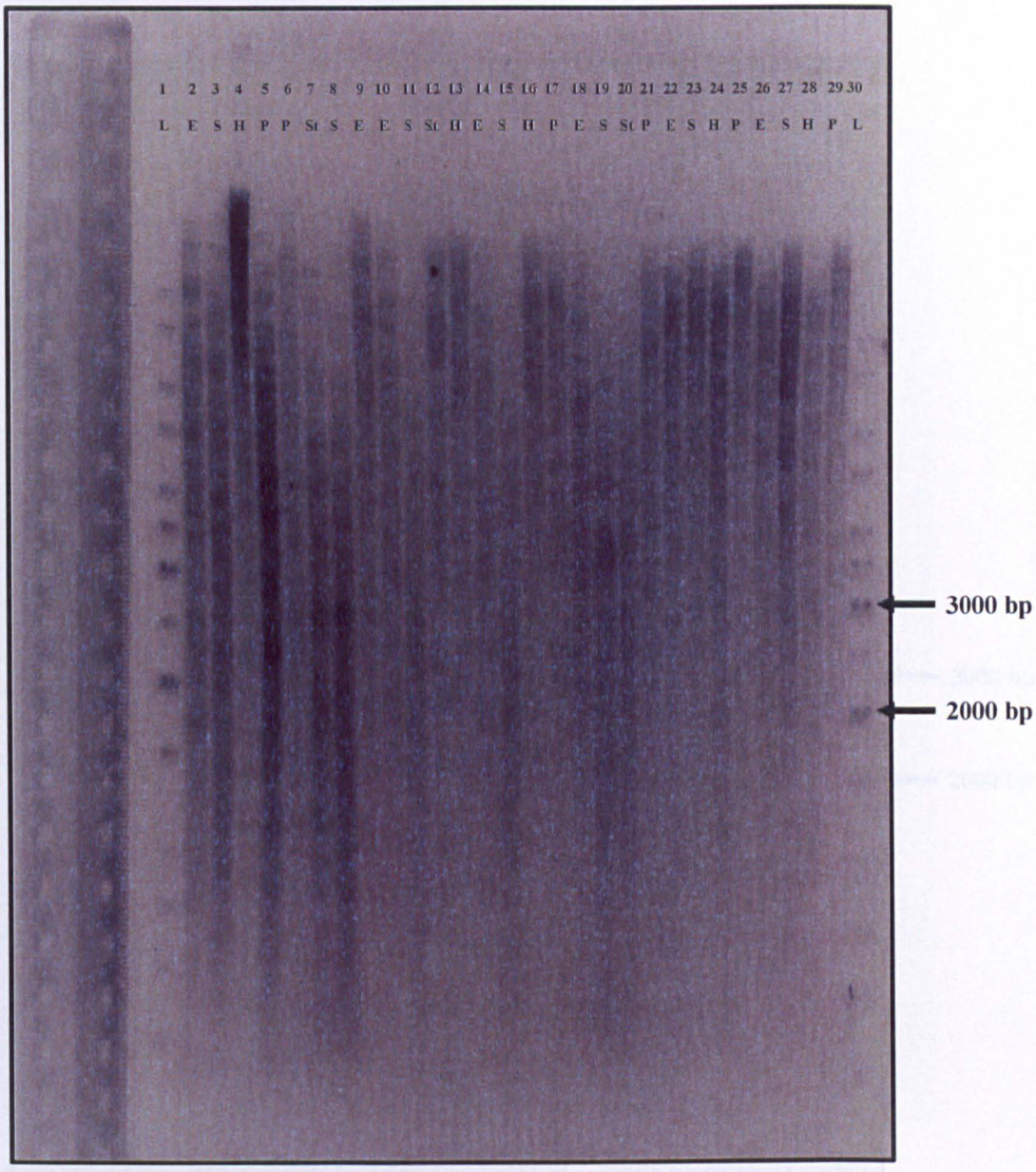


Figure 3.1 Agarose gel (0.9% w/v) of genomic DNA digests of various methanotrophs. Restriction enzymes abbreviations, E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; S, *Sal*I; St, *Sst*I. Lane contents: lanes 1 & 30, 1 kb DNA ladder (GeneRuler); lanes 2-5, *Methylococcus capsulatus* (Bath); lanes 6-9, *Methylosinus trichosporium* OB3b; lanes 10-13, *Methylobacter agile* A20; lanes 14-17, *Methylocystis parvus* OBBP; lanes 18-21, *Methylosinus sporium* 5; lanes 22-25, *Methylomonas methanica* S1; lanes 26-29, *Methylomonas rubra* DNA digests.

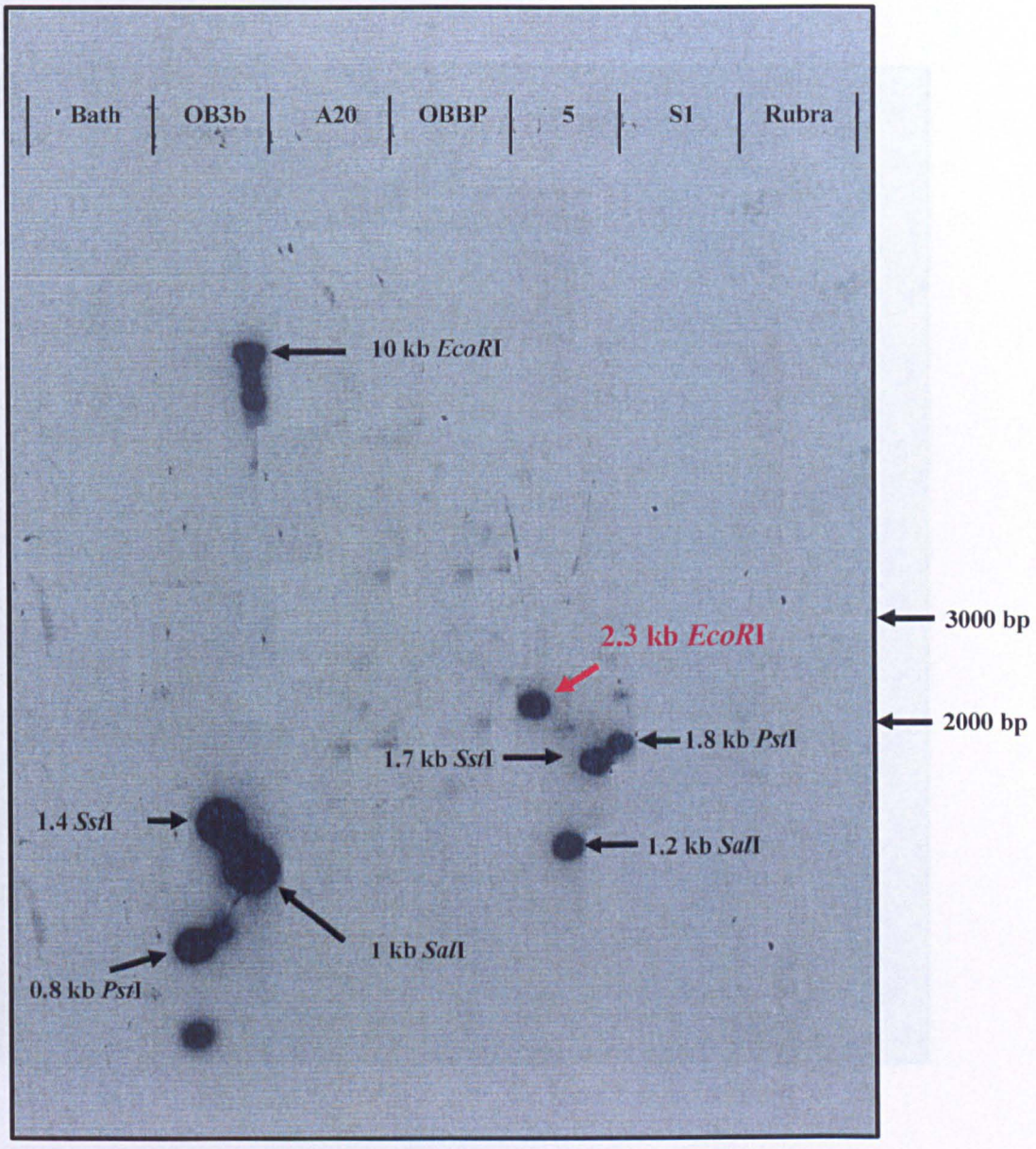


Figure 3.2 Southern blot containing genomic DNA digests from various methanotrophs and probed with *mmoR* from *Ms. trichosporium*. The sizes of the DNA fragments that hybridised to *mmoR* are indicated. Abbreviation; Bath, *Methylococcus capsulatus*; OB3b, *Methylosinus trichosporium*; A20, *Methylobacter agile*; OBBP. *Methylocystis parvus*; 5, *Methylosinus sporium*; S1, *Methylomonas methanica*; and *Rubra*, *Methylomonas*.

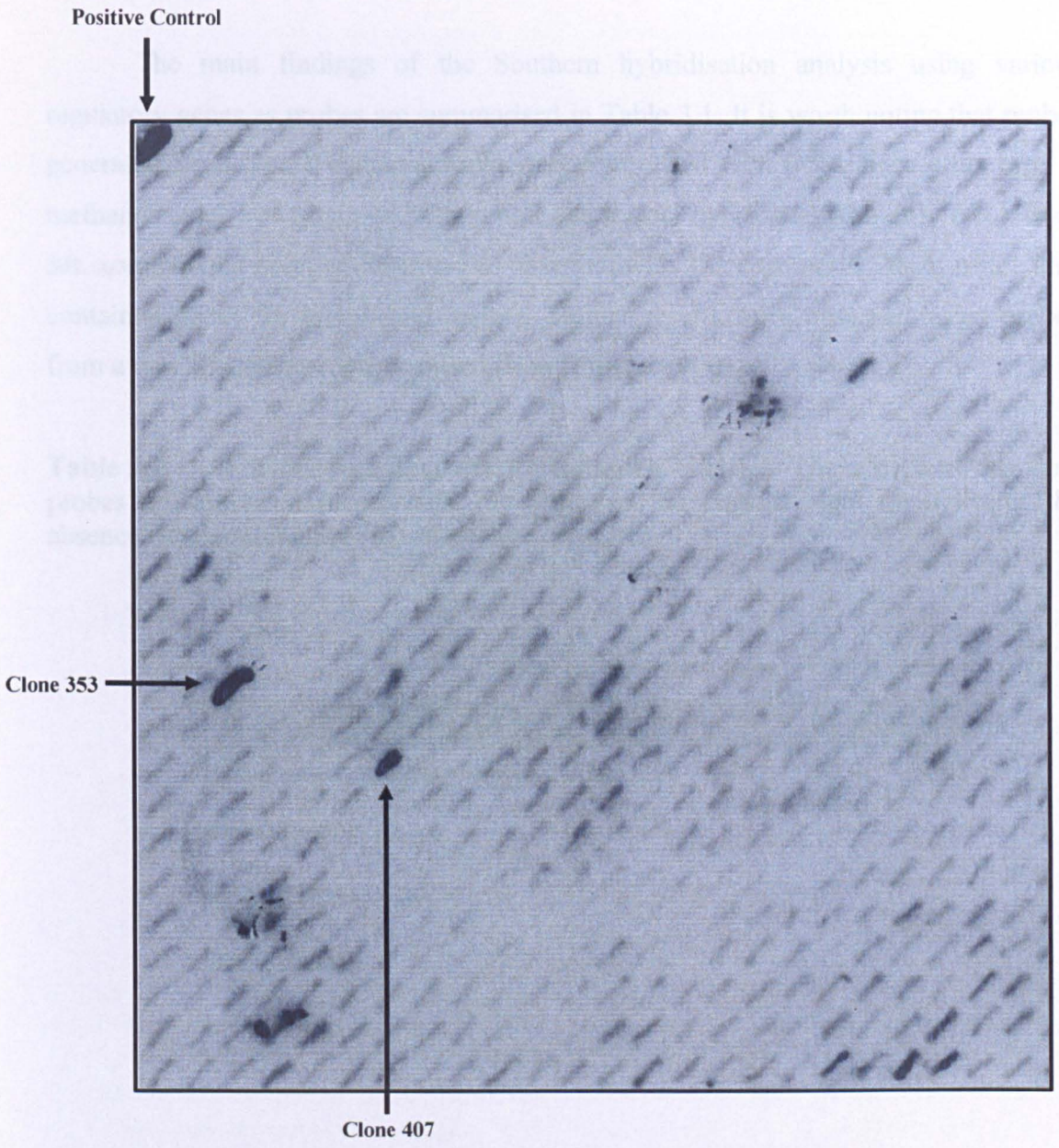


Figure 3.3 Colony blot of 624 *Eco*RI clones in pUC19 containing *Ms. sporium* genomic DNA fragments, hybridised with *mmoR* from *Ms. trichosporium*. The two positive clones (Clones 354 or pHA001 and clone 407 or pHA002) containing *mmoR* homologues are indicated. Plasmid pTJS172 containing the *mmoR* fragment from *Ms. trichosporium* was used as a positive control.

3.2.1 Summary of Southern blottings

The main findings of the Southern hybridisation analysis using various regulatory genes as probes are summarised in Table 3.1. It is worth noting that probes generated from type II methanotrophs only hybridised with DNA from other type II methanotrophs. For example, both *mmoR* and *mmoG* hybridised with only DNA from *Ms. sporium* and no hybridisation was observed with *Mc. capsulatus* DNA, which also contained genes for *mmoR* and *mmoG*. Similar results were observed when probes from a type I methanotroph was used (*mmoQ* and *mmoS*).

Table 3.1 Summary of Southern hybridisation analysis. The source of the gene probes are indicated in brackets. (+) indicates the presence and (-) indicates the absence of a homologue in the respective organism.

	← Type I →				← Type II →		
	Methanotrophic strains						
Gene Probes	**Bath**	***rubra***	**A20**	**S1**	**5**	**OB3b**	**OBBP**
***mmoR* (OB3b)**	-	-	-	-	+	+	-
***mmoG* (OB3b)**	-	-	-	-	+	+	-
***mmoQ* (Bath)**	+	-	-	-	-	-	-
***mmoS* (Bath)**	+	-	-	-	-	-	-

3.2.2 Sequence analysis of pHA001 and pHA002

pHA001 and pHA002 were sequenced as described in Section 2.8. The physical map and the putative *orf*s, as predicted by Clone Manager 5 (version 5.03), are shown in Figure 3.4.

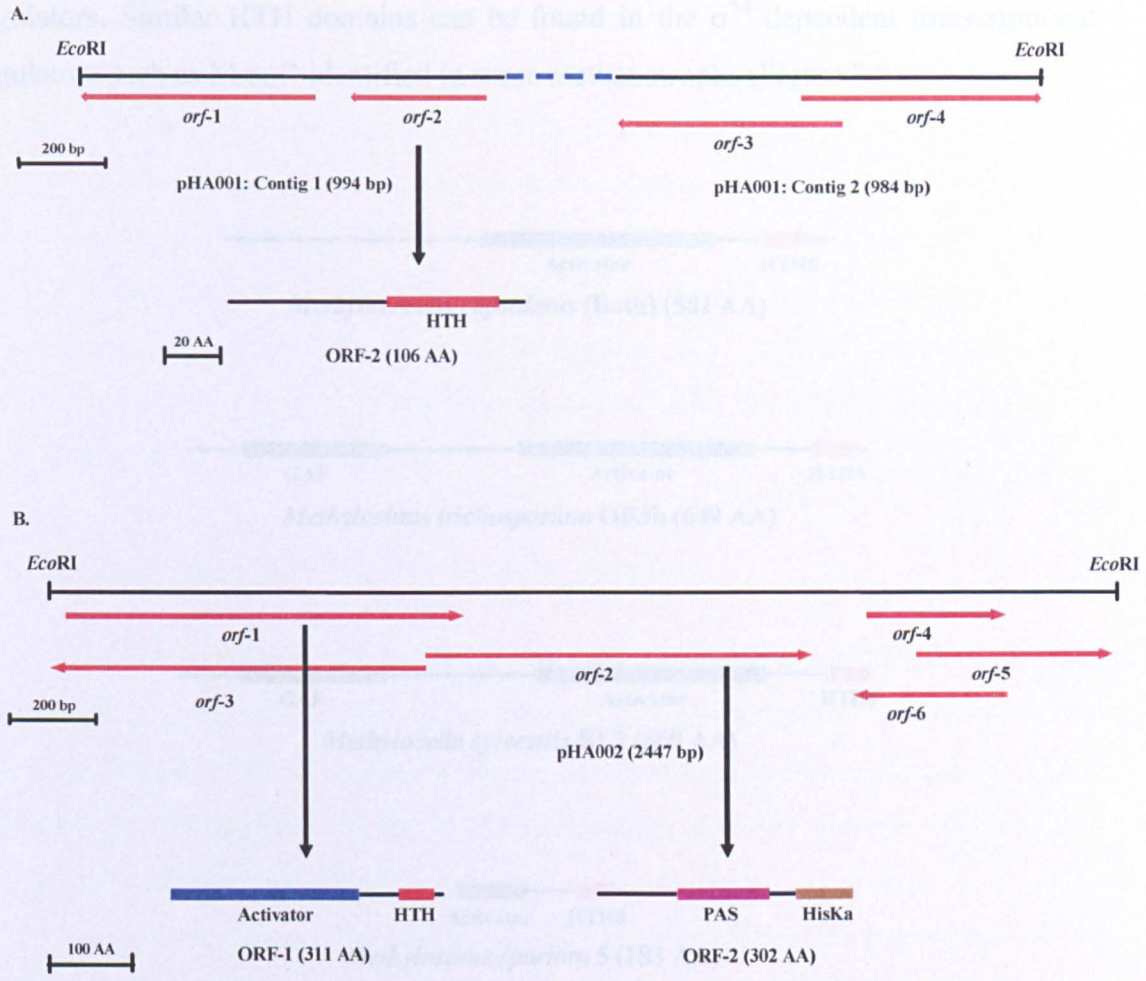


Figure 3.4 Genetic maps of clones pHA001 and pHA002. A) Clone pHA001 consists of two contigs. The unsequenced region between the two contigs is indicated by the blue dotted line. *orf*-2 contains a sequence encoding a putative DNA-binding helix-turn-helix (HTH) domain B) Clone pHA002. The putative protein encoded by *orf*-1 contains an activator domain, which is a σ^{54} interaction domain with a HTH domain. *orf*-2 contains a sequence encoding a putative PAS domain, which may be associated with the input domain of a histidine kinase (HisKa). The *orf*s are indicated by red arrows. Notes: The *orf* and domain boundaries are shown to scale as indicated by the scale bars.

The putative *orf*s were translated to amino acid sequence in all six reading frames and searched for conserved domains using MotifScan (http://au.expasy.org/prosite/). Clone pHA001 was not fully sequenced and thus is represented as two contigs. From the four putative contigs identified in clone pHA001, only *orf*-2 had a conserved domain of a helix-turn-helix (HTH) domains. HTH domains are DNA binding domains, which are typically present in transcriptional

regulators. Similar HTH domains can be found in the σ^{54} dependent transcriptional regulators such as MmoR identified in some methanotrophs (Figure 3.5).

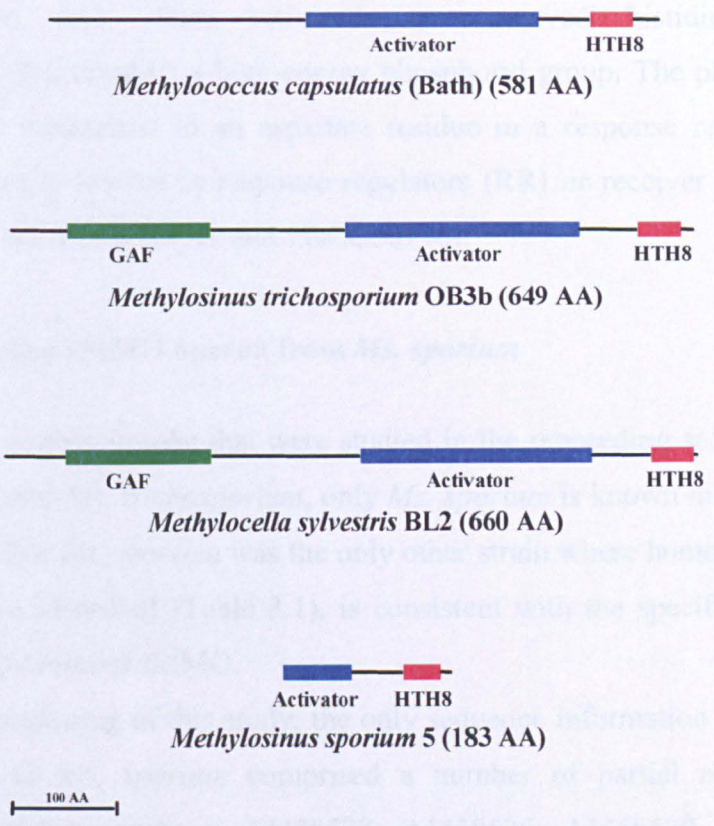


Figure 3.5 Major domain architecture of MmoR, a σ^{54} dependent transcriptional activator, from a number of methanotrophs. GAF domains are typical signalling domain and are evolutionarily linked between diverse transducing phototransducing proteins. The activator domain is typical of a σ^{54}-dependent activator.

Clone pHA002 was fully sequenced and was 2447 bp in length. A number of putative *orf*s were identified from which only two contained identifiable conserved domains. o*rf*-1 encoded a 311 amino acid polypeptide with conserved domains for a typical σ^{54} interaction domain involved in ATP-dependent interaction with σ^{54} for activation of transcription from a σ^{54} promoter. Almost all of these transcriptional activator proteins are associated with HTH domain (Prosite documentation No: PDOC00579), which is also the case in Orf-1. *orf*-2 encoded a 302 amino acid polypeptide with PAS and HisKa conserved domains. Both of these domains are

common features belonging to signal transduction two-component systems, which possess a sensor-kinase protein. PAS domains are usually associated with the sensor protein that regulates a histidine kinase (Prosite documentation No: PDOC50112). Histidine kinase domains are regulated by input signals from sensing domain (PAS and GAF domains) and when activated, a conserved histidine residue is autophosphorylated, creating a high-energy phosphoryl group. The phosphoryl group is subsequently transferred to an aspartate residue in a response regulator domain, which are generally known as response regulators (RR) or receiver domain (Prosite documentation No: PDOC50109 and PDOC50110).

3.3 Cloning the sMMO operon from *Ms. sporium*

Among methanotrophs that were studied in the preceeding section, apart from *Mc. capsulatus* and *Ms. trichosporium*, only *Ms. sporium* is known to produce sMMO and so the fact that *Ms. sporium* was the only other strain where homologues to *mmoR* and *mmoG* were identified (Table 3.1), is consistent with the specific roles of these genes in the regulation of sMMO.

At the beginning of this study, the only sequence information available for the sMMO genes of *Ms. sporium* comprised a number of partial *mmoX* sequences (GenBank accession numbers: AJ458528, AJ458525, AJ458520, AJ458512, and AJ458511). In order to clone and sequence the sMMO operon from *Ms. sporium* and to locate *mmoR* and *mmoG* with respect to the sMMO operon, *mmoX* was PCR amplified from *Ms. sporium* using *mmoX*206F (5'-ATC GCB AAR GAA TAY GCS CG-3') and *mmoX*886R (5'-ACC CAN GGC TCG ACY TTG AA-3') primers described previously (Hutchens *et al.*, 2004). The 718 bp PCR product was radiolabelled as described in Section 2.7.4 and hybridised to a Southern blot containing digests of *Ms. trichosporium* and *Ms. sporium* genomic DNA (Figure 3.6). A number of targets were identified for cloning *mmoX* from *Ms. sporium*. From the initial analysis, it can be seen that two DNA fragments from each of the *Ms. sporium* digest hybridised to *mmoX* probe compared to the *Ms. trichosporium* DNA digest, indicating the possibility of duplicate *mmoX* genes in *Ms. sporium*.

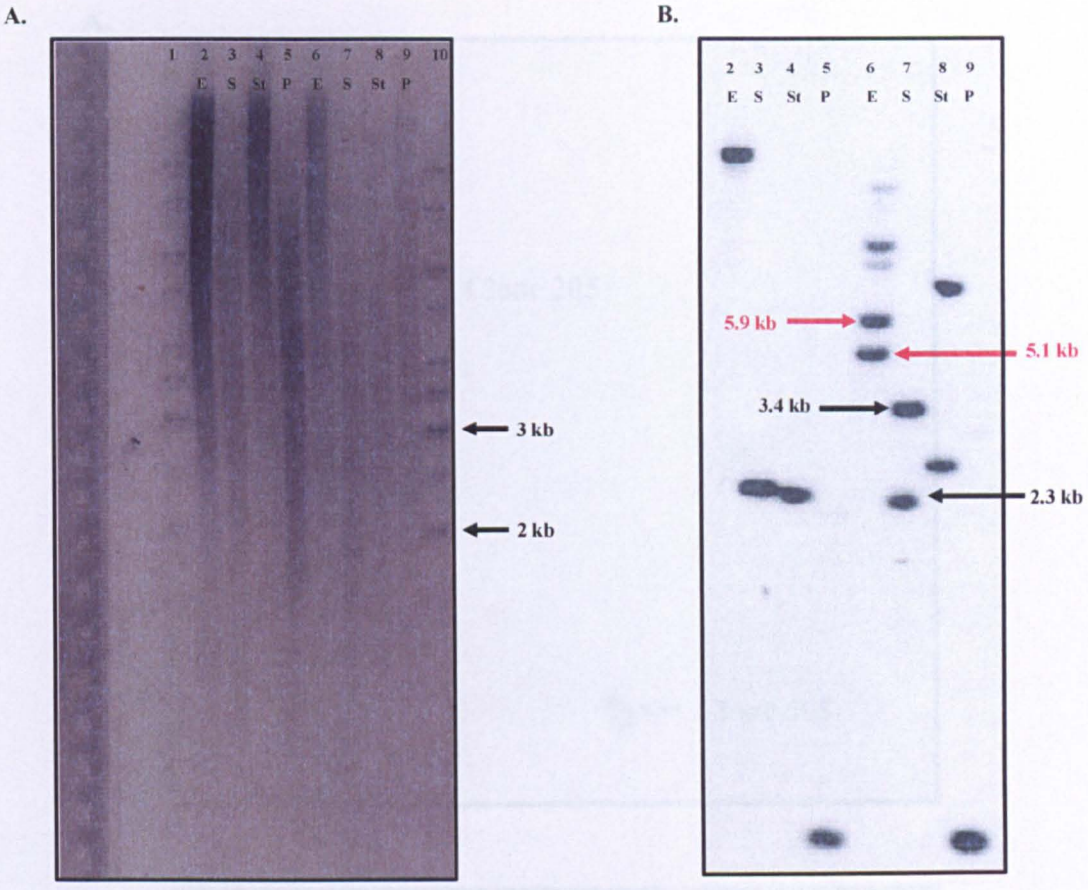
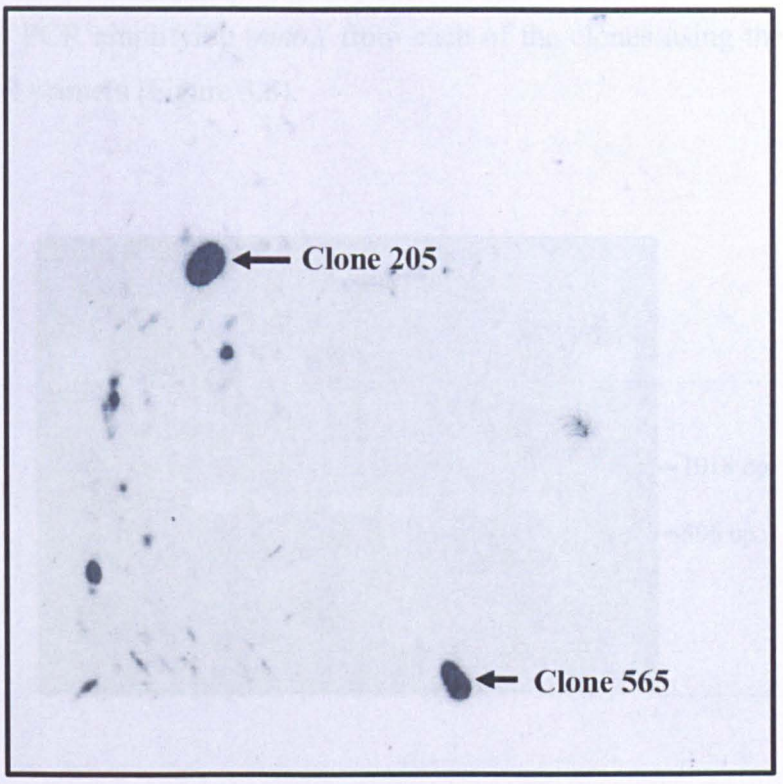


Figure 3.6 A) Agarose (0.9% w/v) gel containing *Ms. trichosporium* and *Ms. sporium* DNA digests. B) A Southern blot hybridised with *mmoX* gene probe. The approximate DNA fragment sizes of *mmoX* homologues are indicated with black arrows from *Sal*I digests and red arrows from *Eco*RI digests. Restriction enzymes abbreviations, E, *Eco*RI; P, *Pst*I; S, *Sal*I; St, *Sst*I. Lane contents: lanes 1 & 10, 1 kb ladder (GeneRuler); lanes 2-5, *Ms. trichosporium*; lanes 6-9, *Ms. sporium* DNA digests.

In order to identify and sequence the possible duplicate *mmoX* genes in *Ms. sporium*, two separate partial clone libraries were constructed as described in Section 2.7.2. The approximate 2.3 kb and 3.4 kb *Sal*I and the approximate 5.1 kb and 5.9 kb *Eco*RI digested genomic DNA fragments, were cloned into pUC19 multiple cloning vector for constructing partial clone libraries. For each library, 625 *E. coli* TOP10F clones were screened by hybridisation using *mmoX* as a gene probe (Figure 3.7). Two *Sal*I clones (clones 205 and 565) were identified, which were designated as pHA003 and pHA004, respectively, and four *Eco*RI clones (clones were 329, 342, 474, and 506) were identified and designated as pHA005, pHA006, pHA007, and pHA008.

A.



B.

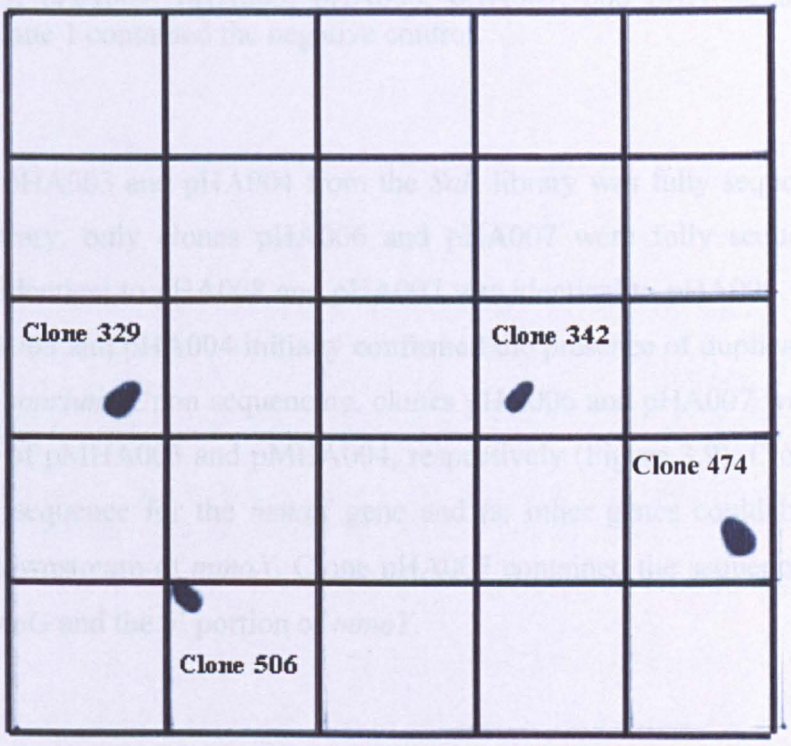


Figure 3.7 Colony blots of A) 625 *Sal*I clones and B) 625 *Eco*RI clones containing *Ms. sporium* genomic DNA fragments, hybridised with *mmoX* gene probe. The clone numbers of the positively identified clones are indicated.

The presence of the *mmoX* gene within the six clones was further confirmed, prior to sequencing, by PCR amplifying *mmoX* from each of the clones using the *mmoX*206F and *mmoX*886R primers (Figure 3.8).

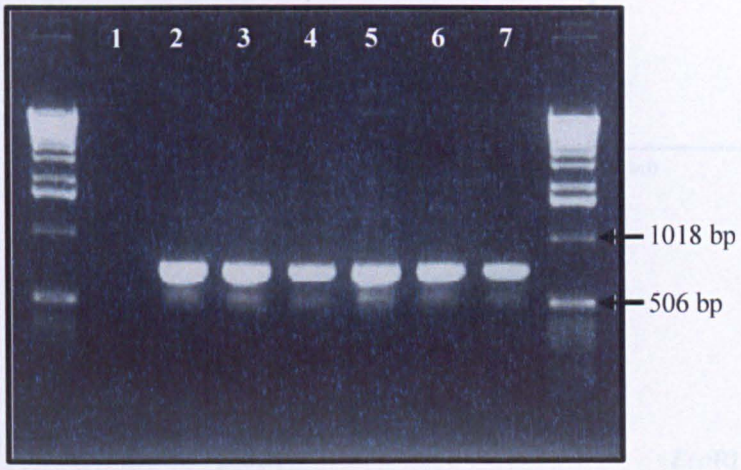


Figure 3.8 Agarose (1 % w/v) gel showing *mmoX* PCR products amplified from clone pHA003, pHA004, pHA005, pHA006, pHA007, and pHA008 in lanes 2-7 respectively. Lane 1 contained the negative control.

Clone pHA003 and pHA004 from the *Sal*I library was fully sequenced. From the *Eco*RI library, only clones pHA006 and pHA007 were fully sequenced since pHA006 was identical to pHA008 and pHA007 was identical to pHA005. Sequencing of clones pHA003 and pHA004 initially confirmed the presence of duplicate copies of *mmoX* in *Ms. sporium*. Upon sequencing, clones pHA006 and pHA007 were found to be extensions of pMHA003 and pMHA004, respectively (Figure 3.9). Clone pHA006 contained the sequence for the *mmoX* gene and no other genes could be identified upstream or downstream of *mmoX*. Clone pHA007 contained the sequence for *mmoX* flanked by *mmoG* and the 5' portion of *mmoY*.

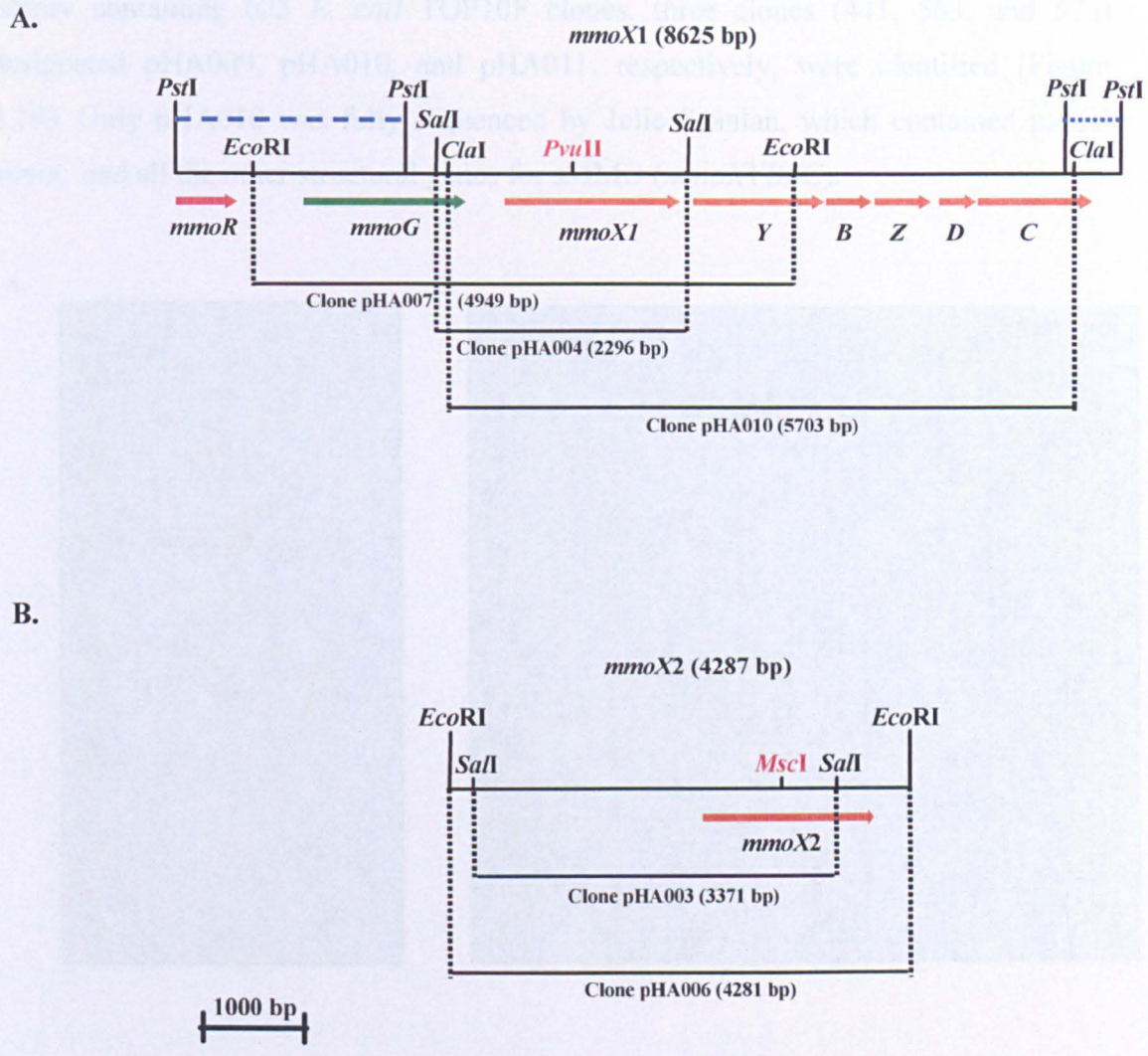


Figure 3.9 Physical maps of the chromosomal region containing the sMMO operons in *Ms. sporium*. A) *mmoX*1 is within an operon containing all the genes for sMMO expression. B) *mmoX*2 is present on its own in the chromosome. Clones used for sequencing are indicated by thick black lanes. Inverse PCR products are indicated by horizontal dotted blue lines between the *Pst*I sites. The unique *Pvu*II and *Msc*I sites within the *mmoX* genes are indicated (Note: Not all restriction sites are indicated). The gene boundaries are shown to scale and are indicated by the scale bar.

In order to target the remainder of the sMMO structural genes and investigate the possible duplication of other sMMO structural genes, another Southern blot was prepared and hybridised with *mmoY* PCR amplified from pHA007 using primers *mmoY*_F (5'-CGC ACG ACC GAC TGG TAT C-3') *mmoY*_R (5'-CGA CTG CGC CGT GAA GAA G-3'). From the Southern blot (Figure 3.10), it was clear that *mmoY* was present as a single copy in the chromosome. Following the screening of a *Cla*I

library containing 625 *E. coli* TOP10F clones, three clones (441, 563, and 573) designated pHA009, pHA010, and pHA011, respectively, were identified (Figure 3.10). Only pHA010 was fully sequenced by Julie Scanlan, which contained partial *mmoC* and all the other structural genes for sMMO (*mmoXYBZC*).

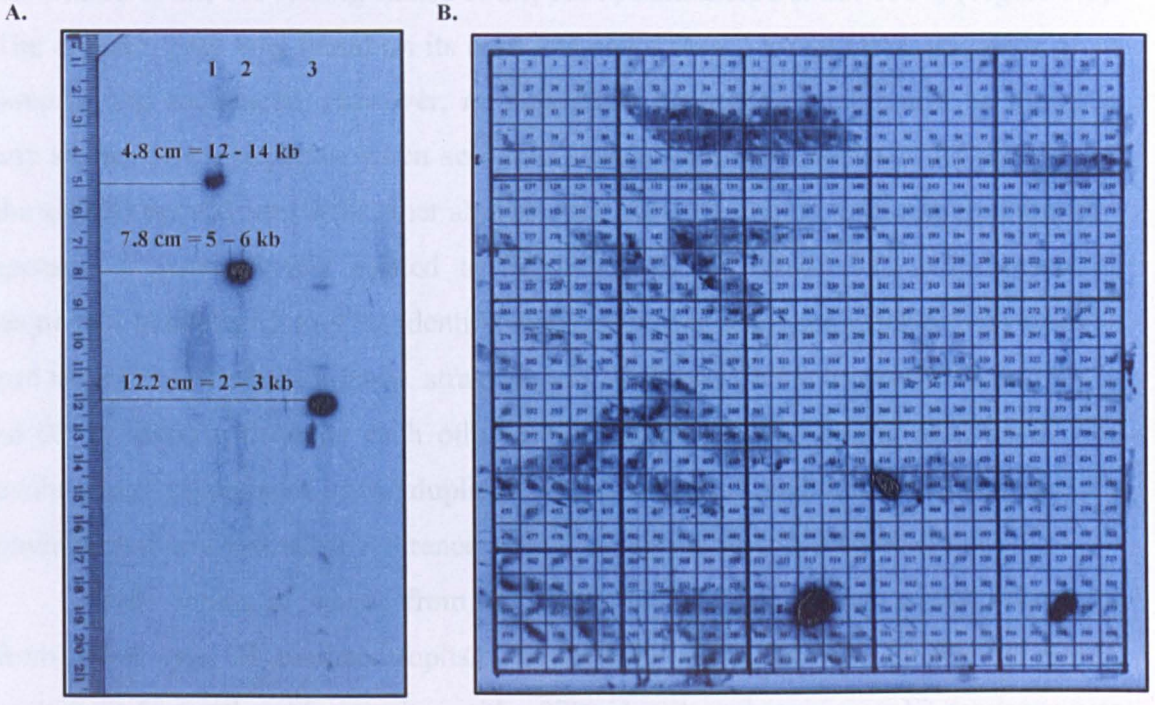


Figure 3.10 A) A Southern blot containing *Ms. sporium* genomic DNA digested with *Bam*HI, *Cla*I, and *Sac*I in lanes 1-3 respectively and probed with *mmoY*. B) A colony blot of 625 *E. coli* TOP10F clones containing *Cla*I fragments of *Ms. sporium* genomic DNA probed with *mmoY*. All three positive clones, pHA009, pHA010, and pHA011 were identical.

Inverse PCR (Section 2.6.1) using primers *mmoC*_F (5'-AAT GGC GTC GCC AAG GGA AC-3'), *mmoC*_R (5'-CCG CCA CCT GCA CAT CGA G-3'), *mmoG*_F (5'-ACG GCT CGA CGA CCT CTG-3'), and *mmoG*_R (5'-GAT TAG TGT CGT GGC CAG GA-3') were used to complete the sequence of *mmoC* and to obtain partial DNA sequence of *mmoR* located 5' of *mmoG*. The *mmoX* gene present in the full sMMO operon was designated as *mmoX*1 and the lone copy of *mmoX* gene designated as *mmoX*2. The sequencing of the sMMO operon including *mmoG* and *mmoR* and the second lone copy of *mmoX* is summarised in Figure 3.9.

3.4 Sequence analysis of the sMMO operon

The *mmoX*1 gene was found in a full operon of sMMO structural genes (*mmoXYBZDC*). The arrangement of the structural genes encoding sMMO enzyme in *Ms. sporium* was identical to those sequenced previously (Cardy *et al.*, 1991b; McDonald *et al.*, 1997; Shigematsu *et al.*, 1999; Stainthorpe *et al.*, 1990) (Figure 1.8). The *mmoX*2 gene was found on its own and more than 2 kb of DNA sequence 5' of *mmoX*2 was sequenced. However, no significant sequence match could be found to any known DNA sequence when searched against GenBank database. Alignments of the sMMO gene cluster with other sMMO gene clusters, clearly demonstrated that *Ms. sporium* is most closely related to *Methylocystis* sp. Strain M, with nucleotide sequences between 81 to 97% identity. The nucleotide sequence identity with *mmoX*1 and *mmoX*2 to *Methylocystis* sp. strain M was 96 % and 88 %, respectively, and 89 % ad 92 % identity between each other at the nucleotide and amino acid level. The evolutionary relatedness of the duplicate copies of *mmoX* was further investigated by phylogenetic analysis using reference *mmoX* sequences from GenBank (Figure 3.11).

Both copies of *mmoX* from *Ms. sporium* branched within *mmoX* sequences from other type II methanotrophs. The *mmoX*1 sequence was closest to *mmoX* sequences from other *Ms. sporium* with ~97% identity, whereas *mmoX*2 sequence was closest to *mmoX* sequences from *Methylocystis*. spp. with ~90 % identity.

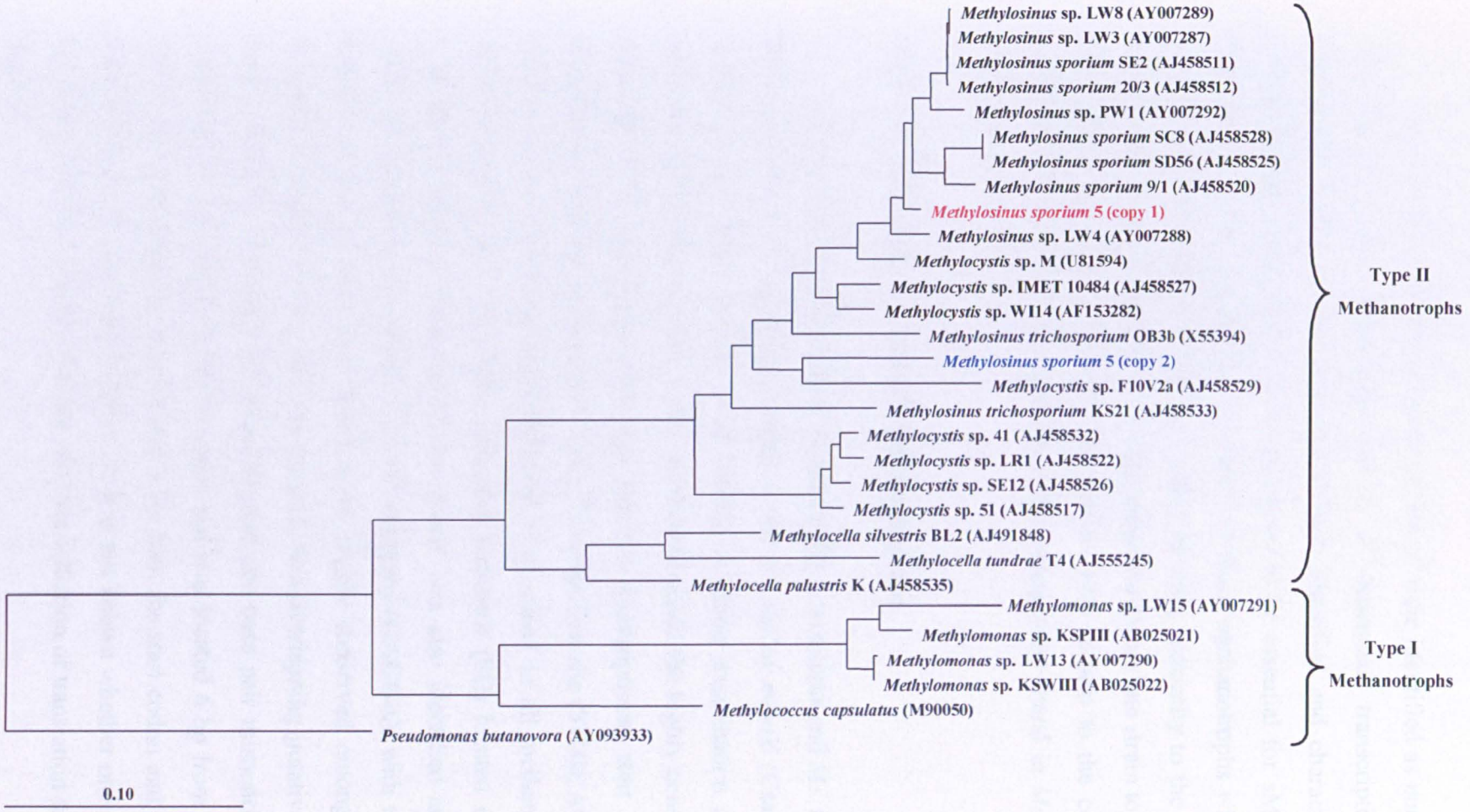


Figure 3.11 Phylogenetic tree of *mmoX* nucleotide sequences. The alignment was based on *mmoX* sequences longer than 1200 bp. The two main clades separating the Type I and Type II methanotrophs are indicated. The related butane monooxygenase (*bmo*) from '*Pseudomonas butanovora*' belonging to the γ-subdivision of proteobacteria was used to root the tree. The accession numbers of the reference *mmoX* sequences are shown in parentheses.

3.4.1 Transcriptional regulators, *mmoG* and *mmoR*

The two *orf*s located upstream of *mmoX* were identified as *mmoG* and *mmoR*, encoding a GroEL homologue and a σ^{54}-dependent transcriptional regulator, respectively. Both of these genes have been identified and characterised in other methanotrophs, where they have been shown to be essential for sMMO expression (Csaki *et al.*, 2003; Stafford *et al.*, 2003). In those methanotrophs where *mmoG* and *mmoR* have been identified, they are found in close proximity to the sMMO operon, however, the exact arrangements of these genes vary from one strain to another (Figure 1.8). The arrangement of *mmoG* and *mmoR* with respect to the complete sMMO operon in *Ms. sporium* was identical to the arrangement found in *Ms. trichosporium* (Stafford *et al.*, 2003).

3.4.2 σ^{54} promoters and ribosomal binding sites

Transcription of the sMMO operon in *Mc. capsulatus* and *Ms. trichosporium* is initiated from a σ^{54} promoter located at the 5' end of *mmoX* (Csaki *et al.*, 2003; Nielsen *et al.*, 1996; Nielsen *et al.*, 1997). A closer examination of the sequence upstream of the *mmoX* genes in *Ms. sporium* revealed the highly conserved sequence resembling σ^{54} recognition sites and putative transcriptional start sites were also identified in both copies of *mmoX*. The σ^{54} recognition site (**TGGCAC-N_5-TTGCW**) (Barrios *et al.*, 1999) has been identified 5' of *mmoX* in all methanotrophic strains analysed (Table 3.2). The Shine-Dalgarno sequence (SD) located upstream of the translation initiation codon (AUG) for *mmoX* was also identified and aligned with other SD sequences for *mmoX*. The SD sequence (**GAGGA**), with the exception of *mmoX*2 of *Ms. sporium* was found to be highly conserved among the sequences analysed. Upstream of the AUG of *mmoX*2, two overlapping putative SD sequences were identified. The first SD sequence had one base pair mismatch to that of the consensus SD sequence for *mmoX* genes and was located 6 bp from the start codon. The second SD sequence was located 3 bp from the start codon and was identical to the consensus SD sequence (Table 3.2). It is not known whether one or both of these SD is involved in ribosome binding and thus initiation of translation from the AUG of *mmoX*2.

Table 3.2 Alignment of the *mmoX* σ^{54} promoters and the SD sequences of various methanotrophs. The -24 and -12 recognition sites for σ^{54} promoter are highlighted in blue and the putative transcriptional start sites in red. The distances between the transcriptional start sites and the initiation codons (AUG) are shown in brackets. The conserved SD sequences are highlighted in green and the distances between the SD sequence and the AUG are indicated in brackets. SD = Shine-Dalgarno. W = A or T. *Ms*, *Methylosinus*; *Mcy*, *Methylocystis*; *M*, *Methylocella*; *Mc*, *Methylococcus*; *Mm*, *Methylomonas*.

Strain	sMMO σ^{54} promoter sequence		SD sequence		GenBank accession no:	Reference
Bacterial consensus	-24 -12 TGGCACNNNNNTTGCW[1]		GAGGA[2]			[1](Barrios *et al.*, 1999) [2](Shine & Dalgarno, 1974)
Ms. sporium (*mmoX*1)	TCAGTGGCACGAGGCTTGCCATAACAATAAGCGTCGT	(143)	TGAGGAGGAAGAAGCATG	(6)	DQ386732	This study
Ms. sporium (*mmoX*2)	ATTACGGCACACACCTTGCTGTGAAAGAACCGACGTC	(133)	TCCAGCGGAGGAATCATG TCCAGCGGAGGAATCATG	(6) (3)	DQ386733	This study
Ms. trichosporium OB3b	CGAGTGGCACAGGCCTTGCCAAATAAGAAGCGTCGAC	(149)	TCAGGAGGAACAAGCATG	(6)	X55394	(Cardy *et al.*, 1991b)
Mcy. strain M	TGACTGGCACGCGCCTTGCCAAATAAGTCGGGTCATC	(148)	GTAGGAGGAACAAGAAATG	(7)	U81594	(McDonald *et al.*, 1997)
M. silvestris BL2	AGCGCGGCGCCACACTTGCTGATAGGGTAGCGCCACA	(97)	CAAGGAGGAGACATATG	(5)	AM072757	(Theisen *et al.*, 2005)
Mc. capsulatus (Bath)	AAAGTGGCACGATCCCTGTAACTAGGTTGTCACGACC	(186)	TACGGAGGAAACAAGTAATG	(8)	AE017282	(Ward, 2004)
Mm. strain KSPIII	ATGCTGGCACGTGTGTTGCAATCTGCCCTGCGAGGCT	(108)	CAACGAGGATTCAATATG	(6)	AB025021	(Shigematsu *et al.*, 1999)
Mm. strain KSWIII	ATACTGGCACACGTGTTGCAATCTGACCACCGAGGCT	(108)	CAACGAGGATTCAATATG	(6)	AB025022	(Shigematsu *et al.*, 1999)

3.5 Transcriptional, biochemical and mutational analysis of MMO

3.5.1 Transcriptional analysis of MMO

To investigate whether the MMO genes in *Ms. sporium* are differentially regulated by copper at the level of transcription as in other methanotrophs, such as *Mc. capsulatus* and *Ms. trichosporium* (Nielsen *et al.*, 1996; Nielsen *et al.*, 1997), it was necessary to carry out RNA dot blotting and RT-PCR.

In order to obtain good quality biomass, expressing pMMO and sMMO grown under high and low copper growth conditions respectively, it was necessary to cultivate *Ms. sporium* in a 5 L fermentor as described in Section 2.2.3. Three separate batch cultures were grown in the 5 L fermentor. The first batch culture contained 1 μM $CuSO_4$ and did not express sMMO. The second batch was grown with no added copper and after 16 hours of growth the cells were expressing sMMO, as detected by the naphthalene assay (Section 2.10.1). The third batch was grown with 5 μM $CuSO_4$ and no sMMO activity was detected (Figure 3.12).

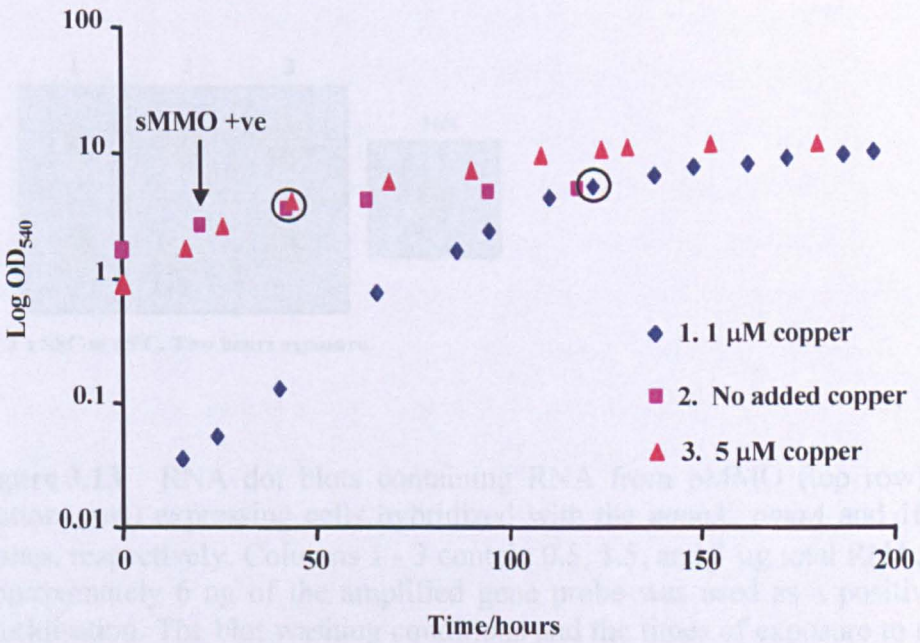


Figure 3.12 Growth curves of *Ms. sporium* grown in a 5 L fermentor under different copper concentrations. The first batch, represented by blue diamonds, was grown in medium containing 1 μM $CuSO_4$. The second batch, represented by purple boxes, was grown in medium containing no added copper. The point at which sMMO activity was first detected is indicated on the graph. The final batch, represented by red triangles, was grown in medium containing 5 μM $CuSO_4$. The points at which cells were harvested for RNA and protein extractions are indicated by circles.

sMMO and pMMO expressing cells were harvested during mid-exponential growth phase from batch 2 and 3, respectively (Figure 3.12). Total RNA and soluble proteins were extracted for subsequent molecular and biochemical analysis.

RNA dot blots were prepared as described in Section 2.7.3, containing various amounts of total RNA extracted from sMMO expressing cells (batch 2) and pMMO expressing cells (batch 3). The RNA dot blots were subsequently probed with *mmoX*, *pmoA*, and 16S rRNA gene probes PCR amplified from *Ms. sporium* using primers previously described (Holmes *et al.*, 1995; Hutchens *et al.*, 2004) (Figure 3.13).

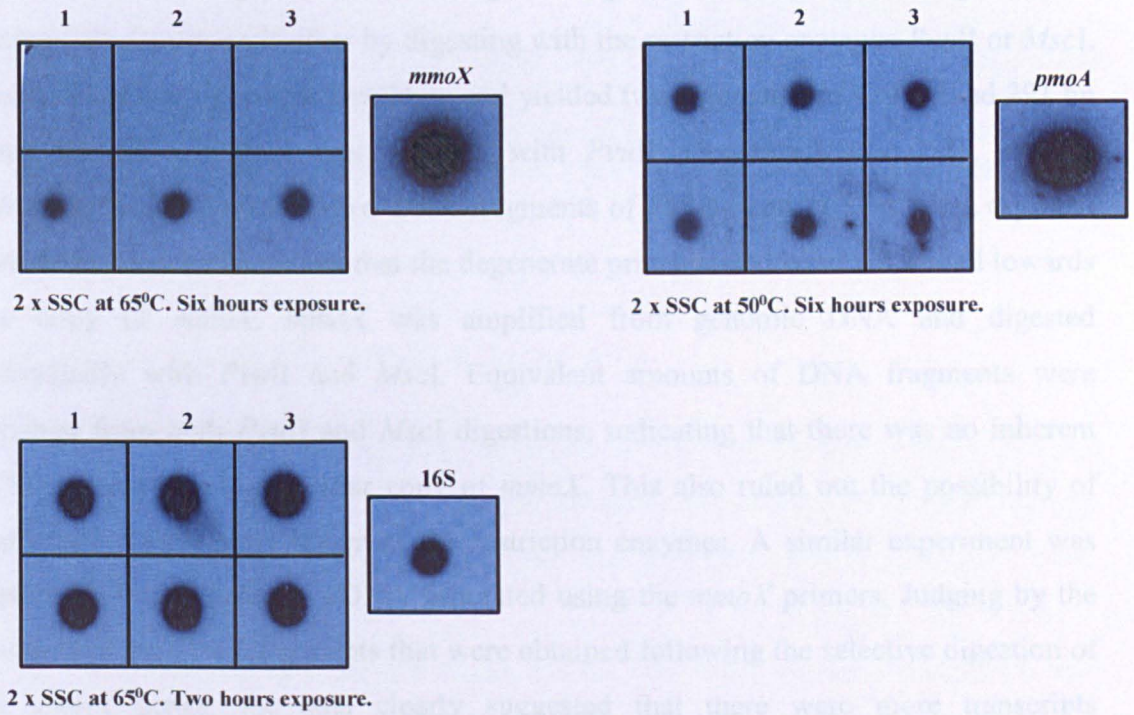


Figure 3.13 RNA dot blots containing RNA from pMMO (top row) and sMMO (bottom row) expressing cells hybridized with the *mmoX*, *pmoA* and 16 rRNA gene probes, respectively. Columns 1 - 3 contain 0.5, 1.5, and 3 μg total RNA, respectively. Approximately 6 ng of the amplified gene probe was used as a positive control for hybridisation. The blot washing conditions and the times of exposure to the x-ray film are indicated below each blot.

The *mmoX* DNA probe hybridized only with RNA extracted from sMMO expressing cells, thus indicating that the transcription of the sMMO, presumably from the σ^{54} promoter, is totally repressed under high copper-to-biomass ratio. The *pmoA*

DNA probe, however, hybridised with RNA extracted from both pMMO and sMMO expressing cells. A separate RNA blot was probed with a 16S rRNA gene probe as a control experiment to examine the extent of RNA degradation of the different RNA samples.

It is noteworthy that the *mmoX* gene probe used for probing the RNA dot blot contained both copies of *mmoX*. Therefore, in order to investigate whether both copies of *mmoX* were transcribed, RT-PCR was performed on RNA extracted from sMMO expressing cultures. The cDNA was synthesised and amplified using the degenerate *mmoX* primers described previously (Hutchens *et al.*, 2004) which yielded a 718 bp product. The PCR products containing the duplicate copies of *mmoX* gene were distinguished from each other by digesting with the restriction enzymes *Pvu*II or *Msc*I. *mmoX*1 contained a unique *Pvu*II site and yielded two fragments of 429 bp and 291 bp when the PCR product was digested with *Pvu*II. Conversely, the PCR product containing *mmoX*2 yielded two DNA fragments of 555 bp and 163 bp when digested with *Msc*I. In order to clarify that the degenerate primers used were not biased towards one copy of *mmoX*, *mmoX* was amplified from genomic DNA and digested individually with *Pvu*II and *Msc*I. Equivalent amounts of DNA fragments were obtained from both *Pvu*II and *Msc*I digestions, indicating that there was no inherent PCR bias towards a particular copy of *mmoX*. This also ruled out the possibility of inefficient digestion by either of the restriction enzymes. A similar experiment was performed in duplicate on cDNA generated using the *mmoX* primers. Judging by the amounts of the DNA fragments that were obtained following the selective digestion of the *mmoX* genes, the data clearly suggested that there were more transcripts corresponding to *mmoX*1 than *mmoX*2 (Figure 3.14). It is noteworthy that although RT-PCR can not be used as an absolute measure for the relative quantification of *mmoX* mRNA *in situ*, it can be used as a crude method for the measurement of the relative abundance of mRNA transcripts *in situ* with the support of the appropriate controls, as used in these experiments.

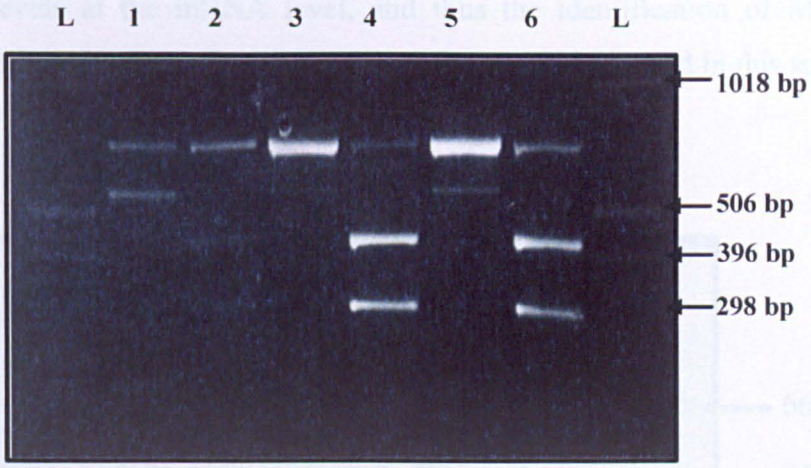


Figure 3.14 RT-PCR. *mmoX* amplified by PCR from genomic DNA using *mmoX*206F and *mmoX*886R primers and digested with *Msc*I (lane 1) and *Pvu*II (lane 2). *mmoX* amplified from cDNA using *mmoX*206F and *mmoX*886R primers and digested with *Msc*I (lane 3 & 5) and *Pvu*II (lanes 4 & 6). L = DNA ladder. Notes: *Pvu*II restriction site is unique to *mmoX*1 and *Msc*I is unique to *mmoX*2.

3.5.2 Biochemical analysis of MmoX expression

The question of whether both copies of *mmoX* were translated into functional hydroxylase was also addressed. Cell-free extracts from sMMO expressing cultures were prepared as described in Section 2.9.1 and separated on a 12.5 % (w/v) SDS-PAGE gel (Figure 3.15).

The α-subunit of the hydroxylase was cut out with a scalpel and digested with trypsin and subsequently analysed by LC-ESI-MS/MS by the Biological Mass Spectrometry and Proteomics Group at Warwick, using three alternative methods. The first method analysed the most abundant peptides for which tandem MS experiments were performed. The second analysis was performed on peptides unique to *mmoX*2 and the third analysis was performed by instructing the software to ignore all peptides common to both copies of *mmoX*. The resulting MS/MS spectra were searched against an in-house database. The most abundant peptides identified were either unique to MmoX1 or common to both MmoX and therefore could not be differentiated. No peptides unique to MmoX2 were identified. Therefore it is not clear whether MmoX2 is assembled into active enzyme or whether there is not enough of the protein present,

due to the low levels at the mRNA level, and thus the identification of MmoX2 polypeptides are below the detection limits of the LC-ESI-MS/MS used in this study.

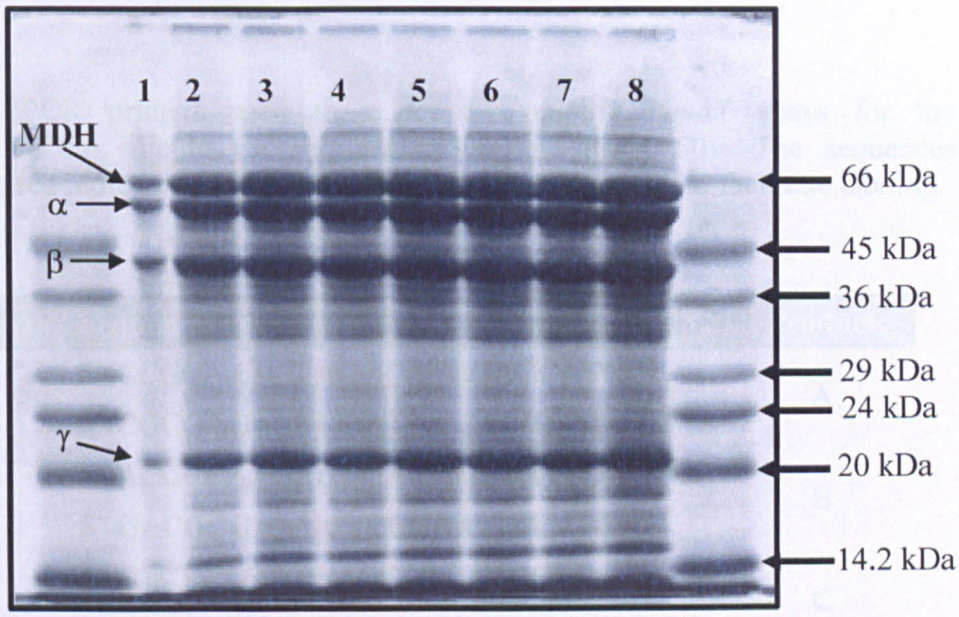


Figure 3.15 SDS-PAGE (12.5 % w/v) containing varying amounts of cell-free extracts from *Ms. sporium* expressing sMMO. Lanes 1-8 contain increasing amounts of crude cell-free extracts from 42 μg to 196 μg. A Dalton VII molecular marker was used either side of the samples and their sizes are indicated on the sides.

3.5.3 Marker-exchange mutagenesis

The data presented in Section 3.5.1 suggest that both copies of *mmoX* are transcribed. However, from the biochemical analysis there is no clear evidence that the *mmoX*2 gene product is incorporated into a functional hydroxylase, thus raising the possibility that the *mmoX*2 gene may not encode a protein that is functional in this strain. In order to definitively conclude whether the duplicate copies of *mmoX* were functional and thus play a role in the formation of an active sMMO, knock-out mutants were constructed individually on both copies of *mmoX* by marker-exchange mutagenesis. The construction of *mmoX* mutants would then allow us to answer the question of whether the expression of one copy of *mmoX* can compensate for the deletion of the other copy. This section describes the molecular approaches used to

construct the suicide vectors required for replacing a portion of the *mmoX* genes with a gentamicin cassette and thus the construction of *mmoX* mutant strains.

Two regions flanking *mmoX*1 and *mmoX*2 were amplified by PCR using primers listed in Table 3.3 and were designated products A-to-D, respectively (Figure 3.16A & B).

Table 3.3 PCR primers used to selectively amplify *mmoX* genes for the construction of the suicide vectors, pMHA500 and pMHA501. The sequences highlighted in red indicate restriction sites introduced deliberately to facilitate cloning.

Primers	Sequence	Product
*mmoX*1**AF**	5'-CCGCAA**TCTAGA**CATCGAGCATC-3'	A
*mmoX*1**AR**	5'-CGCGAGACT**GCATGC**CATGC-3'	
*mmoX*1**BF**	5'-ACGCCAAGAC**GCATGC**CTATTG-3'	B
*mmoX*1**BR**	5'-GACGCG**AAGCTT**CGAATGAAG-3'	
*mmoX*2**CF**	5'-CTCTC**TCTAGA**GCGTCATCGTC-3'	C
*mmoX*2**CR**	5'-CGCATT**GCATGC**GAGACTGATAGG-3'	
*mmoX*2**DF**	5'-CATATG**GCATGC**GGCTATCAGAC-3'	D
*mmoX*2**DR**	5'-CGAGTCTGT**AAGCTT**GAGAGTAGC-3'	

(Note: Due to the high similarity between the two copies of *mmoX* genes at the DNA level, it was difficult to design primers to selectively amplify a DNA fragment from within the *mmoX* gene. Fortunately, the DNA sequencing flanking the *mmoX* genes had no significant sequence similarity. Therefore, the respective *mmoX* genes were selectively amplified by designing one primer internal and the other external to the *mmoX* gene). In order to facilitate cloning of these products into the cloning vector, pK18mob, *Xba*I and *Sph*I sites were introduced into the forward and reverse primers respectively of product A and C, and *Sph*I and *Hin*dIII sites into the forward and reverse primers respectively of product B and D (Table 3.3).

Product A and B, and C and D were cloned into pK18mob via *Xba*I and *Hin*dIII sites to give the intermediate construct pMHA500.1 and pMHA501.1, respectively. A 913 bp *Sph*I fragment from p34S-Gm containing the gentamicin resistance (Gm^R) gene was cloned via the *Sph*I site between product A and B, and C and D to give the final constructs pMHA500 and pMHA501, respectively (Figure

3.16C). pMHA500 and pMHA501 were used to transform *E. coli* S17.1 λ*pir* by electroporation (Herrero *et al.*, 1990). This was then used as a donor strain for conjugation of the two suicide plasmids into *Ms. sporium*. Conjugations were carried out using the method described in Section 2.5.1. The knock-out mutant strains were isolated through replica plating of the respective transconjugants onto NMS agar plates containing gentamicin (5 μg ml^{-1}) alone and gentamicin (5 μg ml^{-1}) plus kanamycin (15 μg ml^{-1}). From the 40 transconjugants screened, more than 50 % were a result of a double homologous recombination event. Isolation of *mmoX*1 and *mmoX*2 mutants strains were designated as *Ms. sporium* Δ*mmoX*1 and Δ*mmoX*2 strains, respectively.

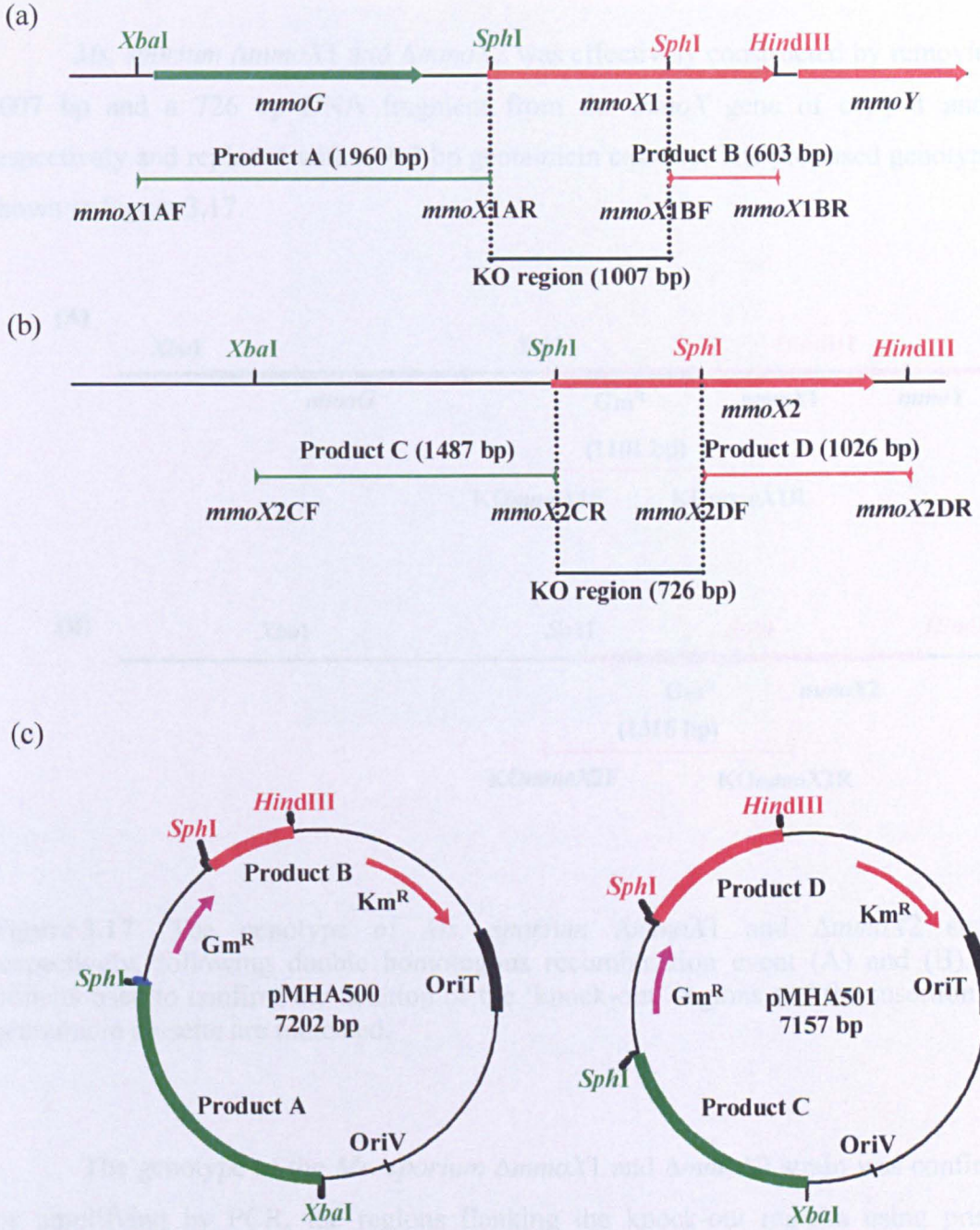


Figure 3.16 Strategy for constructing *Ms. sporium* Δ*mmoX*1 and Δ*mmoX*2 strains. (a) & (b) *mmoX*1 and *mmoX*2 and flanking DNA regions. Primers used to amplify the flanking regions of *mmoX*, yielding products A-to-D, are indicated. Restriction sites highlighted in green and orange were introduced by PCR to facilitate cloning (See Table 3.3). The region between the *Sph*I sites is the 'knock-out' region and is indicated by the dotted lines. (c) The suicide plasmid constructs pMHA500 and pMHA501 used to knock-out *mmoX*1 and *mmoX*2, respectively.

3.5.4 Confirmation of Δ*mmoX*1 and Δ*mmoX*2 genotype

Ms. sporium Δ*mmoX*1 and Δ*mmoX*2 was effectively constructed by removing a 1007 bp and a 726 bp DNA fragment from the *mmoX* gene of copy 1 and 2, respectively and replaced with a 913 bp gentamicin cassette. The proposed genotype is shown in Figure 3.17.

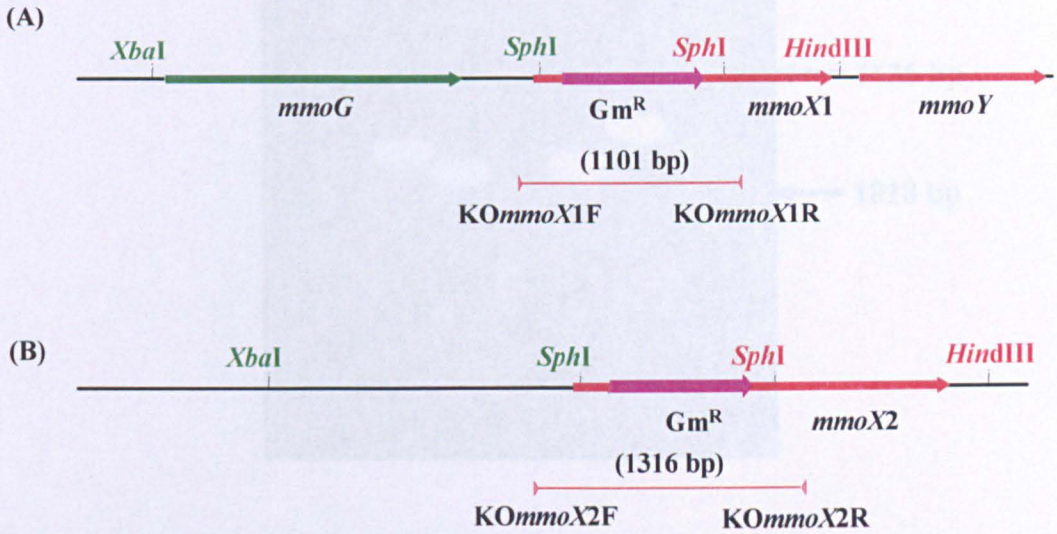


Figure 3.17 The genotype of *Ms. sporium* Δ*mmoX*1 and Δ*mmoX*2 strains, respectively, following double homologous recombination event (A) and (B). The primers used to confirm the deletion of the 'knock-out' regions and the insertion of a gentamicin cassette are indicated.

The genotype of the *Ms. sporium* Δ*mmoX*1 and Δ*mmoX*2 strain was confirmed by amplifying by PCR, the regions flanking the knock-out regions using primers KO*mmoX*1F (5'-CAC GTT CGG ATC TGC GAC TG-3') KO*mmoX*1R (5'-TTC GCC TCG AAC CAC TCC TG-3') to confirm knock-out of *mmoX*1, and primers KO*mmoX*2F (5'-CTG ACA CCG CAT GAT AGC TTC G-3') and KO*mmoX*2R (5'-CAG CGG TTC CAG GTC TTC AC-3') to confirm knock-out of *mmoX*2. The differences in length of the PCR products obtained from the wild-type and mutant strains confirmed the chromosomal deletion of the *mmoX* gene and the insertion of the gentamicin cassette (Figure 3.18).

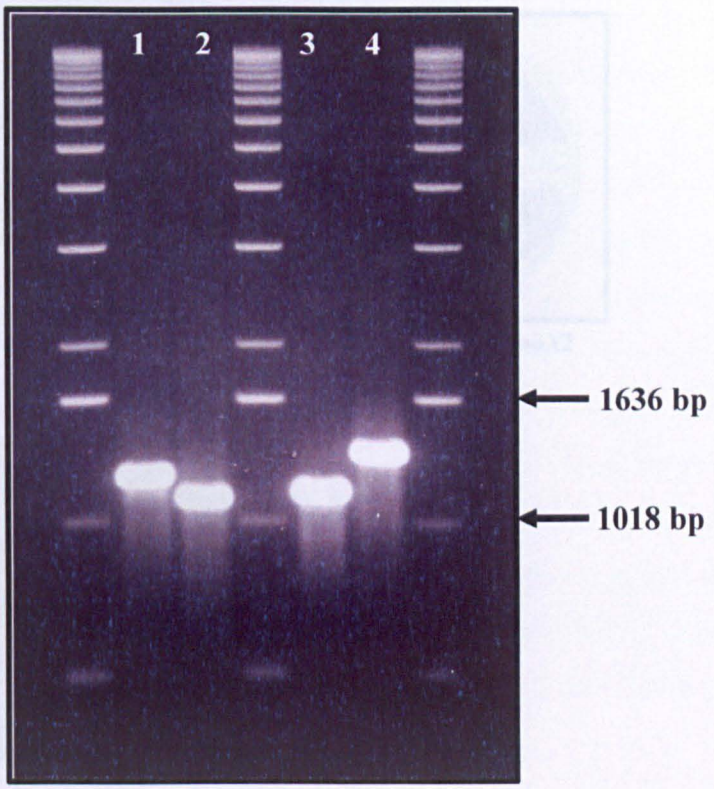
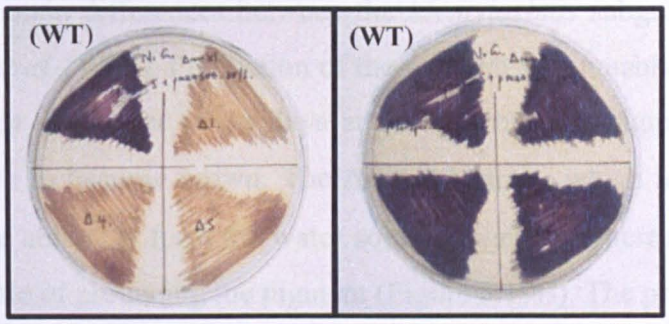


Figure 3.18 Agarose gel (1 % w/v) confirming the genotype of *Ms. sporium* Δ*mmoX*1 and Δ*mmoX*2 mutant strain. The KO*mmoX*1 primers were used to amplify *mmoX* from genomic DNA extracted from wild-type [lane 1 (1195 bp)] and Δ*mmoX*1 [lane 2 (1104 bp)] strain. The KO*mmoX*2 primers were used to amplify *mmoX* from genomic DNA extracted from wild-type [lane 3 (1129 bp)] and Δ*mmoX*2 [lane 4 (1316 bp)] strain. The difference in PCR product length reflects the chromosomal deletion of *mmoX* and the insertion of a gentamicin cassette. A 1 kb DNA ladder was used (Invitrogen).

3.5.5 Phenotypic characterisation of *Ms. sporium* Δ*mmoX*1 and Δ*mmoX*2 strains

The ability of the mutant strains to oxidise methane using the sMMO enzyme was assessed using the naphthalene plate assay described in Section 2.10.1 (Figure. 3.19A). *Ms. sporium* Δ*mmoX*1 strain was unable to oxidise naphthalene indicating loss of sMMO activity, whereas *Ms. sporium* Δ*mmoX*2 strain retained wild-type sMMO activity and was thus able to oxidise naphthalene.

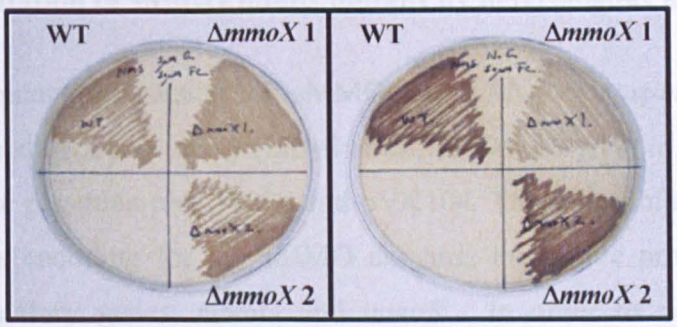
(A)



Ms. sporium Δ*mmoX*1 *Ms. sporium* Δ*mmoX*2

(B)

(C)



(pMMO) (sMMO)

Figure 3.19 Phenotypic characterisation of *Ms. sporium* Δ*mmoX*1 and Δ*mmoX*2 mutant strains (A) The sMMO activity of *Ms. sporium* Δ*mmoX*1 and Δ*mmoX*2 strains analysed using the naphthalene plate assay. For each mutant strain, three transconjugants resulting from double homologous recombination events were tested and *Ms. sporium* wild-type strain was used as a positive control. sMMO naphthalene positive colonies appeared purple. (B) The pigmentation profiles of *Ms. sporium* wild-type and Δ*mmoX*1 and Δ*mmoX*2 strains grown on normal NMS medium and (C) NMS medium containing excess Fe-EDTA (5 μM) and with no added copper (sMMO expressing condition) and excess copper (5 μM $CuSO_4$) (pMMO expressing condition). The colonies producing the water soluble pigment appeared brown.

Ms. sporium has the ability to form a brown-black water soluble pigment, which is one of the main differences between the *Methylosinus* subgroups (Anthony, 1982; Whittenbury *et al.*, 1970). Production of the pigment was notably apparent after growth on NMS agar plates for 10-14 days at 30 °C when the pigment production caused the agar plate to become brown. The Δ*mmoX*1 strain, which had lost sMMO activity, also lost the ability to form the water soluble pigment, whereas the Δ*mmoX*2 strain was still capable of producing the pigment (Figure 3.19B). The production of the pigment initially seemed to be related to the expression of sMMO. However, it was noted by Whittenbury *et al*, (1970) that the pigmentation was only apparent on iron-deficient medium. This was confirmed by growing *Ms. sporium* and the mutant strains on NMS plates containing excess iron conditions. Under excess iron and pMMO expressing conditions, none of the strains produced the water soluble pigment, whereas under iron excess and sMMO expressing conditions, only the sMMO expressing strains (i.e. The wild-type and Δ*mmoX*2 strains) produced the water soluble pigment (Figure 3.19C).

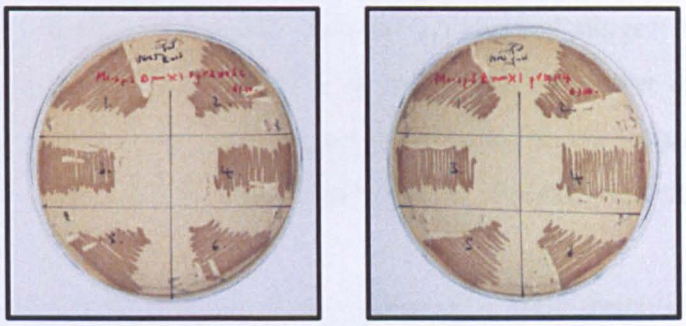
3.6 Complementation of sMMO minus mutant by heterologous expression

The recombinant expression of sMMO from *Ms. trichosporium* and *Mc. capsulatus* has been shown previously (Lloyd *et al.*, 1999a; Lloyd *et al.*, 1999b) using the broad-host range plasmids pVK100Sc and pVK104. These plasmids contained all the structural genes encoding for the sMMO enzyme, the native promoter and the transcriptional regulatory genes, *mmoG* and *mmoR*. In order to demonstrate the complementation and the heterologous expression of sMMO in *Ms. sporium*, plasmids pVK100Sc and pVK104 were conjugated into *Ms. sporium* Δ*mmoX*1 strain individually. Six transconjugants from each conjugation were selected on replica copper minus NMS agar plates containing gentamicin (10 μg ml^{-1}) and kanamycin (15 μg ml^{-1}).

3.6.1 Characterisation of heterologous sMMO expression

Ms. sporium Δ*mmoX*1 strains containing plasmid pVK100Sc and pVK104, respectively, were all expressing active sMMO as judged by the naphthalene plate assay and the ability to secret the water soluble pigment causing the discolouration of

the agar plates. The intensity of the purple colour formed from the naphthalene plate assay and the discolouration of the agar plates from the production of the water soluble pigment, were low compared to that of the wild-type strain indicating low level expression of the recombinant sMMO (Figure 3.20).



Δ*mmoX*1:pVK100Sc **Δ*mmoX*1:pVK104**

Figure 3.20 *Ms. sporium* Δ*mmoX*1 strains containing the sMMO expression plasmids, pVK100Sc and pVK104 respectively. The production of the water soluble pigment in these strains can be seen from the slight discoloration of the colonies and agar plates (Compare with figure 3.19B).

3.7 Discussion

The data presented in this study indicate that the transcription of sMMO operon in *Ms. sporium* is regulated by copper from a copper-regulated σ^{54} promoter and is similar to those studied previously (Nielsen *et al.*, 1996). However, contrary to the data presented previously (Nielsen *et al.*, 1997) on the copper-dependent reciprocal transcriptional regulation of MMO, data presented here suggest that transcription of the *pmoCAB* operon in *Ms. sporium* is not regulated by copper ions. In addition, the regulatory genes, *mmoG* and *mmoR*, have been cloned and sequenced and duplicate copies of *mmoX* gene have been identified and characterised at the molecular and biochemical level.

The Southern hybridisation screenings of a wide range of methanotrophs for the regulatory genes *mmoG* and *mmoR* indicated that they are specifically present in sMMO expressing methanotrophs. Indeed, homologues of *mmoG* and *mmoR* could not be found in any other strains. Since this study, the sMMO operon and the regulatory genes from another sMMO expressing methanotroph, *M. silvestris* BL2 has been cloned and sequenced (Theisen *et al.*, 2005). This gives us further evidence that the regulatory genes *mmoG* and *mmoR* are specifically present in sMMO expressing methanotrophs and that they are key players in copper-dependent regulation of the transcription from the *mmoX* σ^{54} promoters. The role of *mmoG* and *mmoR* in transcription initiation has been further characterised in *Mc. capsulatus* and will be discussed in Chapter 5.

The two-component sensor-regulatory genes, *mmoS* and *mmoQ* respectively, present in *Mc. capsulatus,* could not be identified in any other methanotrophs. These data coupled with the data obtained from transposon mutagenesis experiments (Chapter 6), where a mutant in *mmoQ* was isolated, which was still capable of expressing sMMO, suggest that these genes may not be involved in copper-mediated transcriptional regulation as hypothesised previously (Figure 1.11) (Csaki *et al.*, 2003). Despite not identifying homologues of *mmoS* and *mmoQ* via Southern probing approach, two clones, pHA001 and pHA002, were identified from the size fractionated *Eco*RI library containing *Ms. sporium* DNA probed with *mmoR*. pHA001 contained a putative HTH domain, which is a DNA binding domain of a typical transcriptional regulator. pHA002 contained two putative *orf*s in the same transcriptional orientation with conserved domains resembling a typical two-component sensor regulatory

system. The gene (*orf*-2) encoding the sensor protein contained conserved domains indicative of a histidine kinase (HK) and a PAS domain. The gene (*orf*-1) encoding the regulatory protein had typical domain architecture for a σ^{54}-dependent transcriptional regulator, such as MmoR, with a σ^{54} interaction domain and an HTH domain. It is documented that some subgroups of σ^{54} transcriptional regulators operate as a response regulator for two-component regulatory systems (Shingler, 1996), where a conserved aspartate residue in the N-terminal domain of the regulator is activated by phosphorylation via a histidine residue on an HK. In an alignment of the σ^{54} interaction domains of MmoR, including the partial MmoR amino acid sequence from *Ms. sporium*, similar conserved aspartate residues were identified.

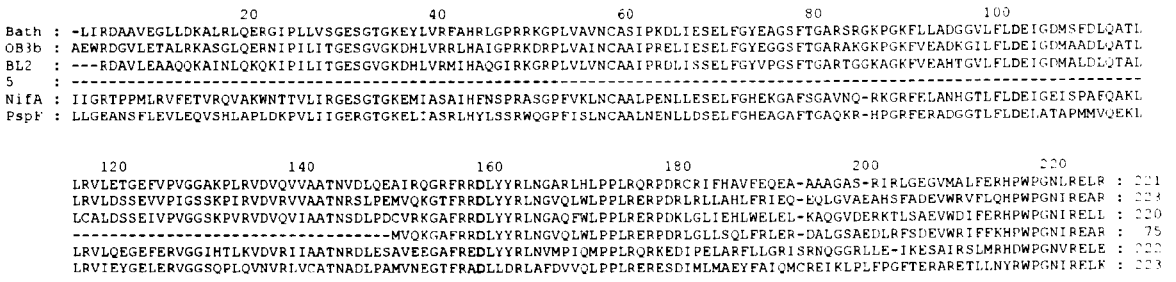


Figure 3.21 Alignment of interaction domains of σ^{54} transcriptional activators. Interaction domains of MmoR from *Mc. capsulatus* (Bath), *Ms. trichosporium* (OB3b) and *M. silvestris* (BL2) were used together with interaction domains of NifA from *Mc. capsulatus* (MCA0764) and PspF, a interaction domain for phage-shock protein (psp) operon in *Escherichia coli*, all of which are driven by a σ^{54} promoter. The conserved aspartate residues are highlighted in blue.

ORF-1 in pHA002 seems to be operating as a response regulator for a two-component regulatory system for which the environmental signal it detects is unknown. Since pHA002 was identified as a result of a non-specific hybridisation with *mmoR* and with the identification of a conserved aspartate residue within the σ^{54} interaction domain, it could be speculated that MmoR is the response regulator of a two-component systems. The sensor protein responsible for MmoR activation via phosphorylation, in response to copper concentration, is not known and will require further investigation.

The tight regulation of the transcription from the *mmoX* σ^{54} promoter in response to copper ions has been shown in *Mc. capsulatus* and *Ms. trichosporium*

(Csaki *et al.*, 2003; Nielsen *et al.*, 1996). Similar tight transcriptional regulation from the *mmoX* σ^{54} promoter was observed in *Ms. sporium*, using RNA dot blotting, where no mRNA corresponding to *mmoX* could be detected under pMMO expressing conditions. As in other methanotrophs that have an inducible sMMO, transcription of the sMMO operon seems to be regulated by a unique copper-switch.

Sequence analysis of the promoter regions indicate that the pMMO operon is transcribed from a σ^{70}-dependent promoter, although there are conflicting data concerning the transcription profile of the pMMO operon under different copper conditions (Choi *et al.*, 2003; Nielsen *et al.*, 1997; Stolyar *et al.*, 2001). In the results from *Ms. sporium* presented above, RNA dot blots hybridised with *pmoA* indicated that transcription of the pMMO operon is not repressed under low copper or sMMO expressing conditions. Almost equal intensity hybridisation signals were observed with RNA extracted from both sMMO (no added copper) and pMMO (5 μM copper) expressing cells. Similar results were observed with RNA from *Mc. capsulatus* (Chapter 5). These data suggest that the MMO enzymes are not regulated by a common copper-switch and that the enzymes are regulated at different levels. The sMMO enzyme is strictly regulated at the level of transcription, whereas the pMMO enzyme seems to be regulated at a level of post-transcription. The data presented here on the pMMO transcription profile are similar to the results obtained by Stolyar *et al.*, (2001) using *Mc. capsulatus*, where they observed less than two-fold increase in activity of XylE under high copper (20 μM copper) conditions using chromosomal gene fusions with both pMMO σ^{70} promoter of copy 1 and copy 2. The slight increase in XylE activity under high copper conditions could be explained due to the increase in growth rate under pMMO expressing conditions compared to that of sMMO expressing conditions.

The duplication of genes encoding key metabolic enzymes such as the particulate methane monooxygenase in methanotrophs and ammonium monooxygenase in nitrifiers is well documented (Arp *et al.*, 2002; Stolyar *et al.*, 1999). In *Mc. capsulatus*, there are two complete *pmoCAB* operons encoding for pMMO, and a third copy of *pmoA*. A comprehensive phylogenetic analysis of sMMO and other soluble diiron monooxygenases reviewed in Leahy *et al.*, (2003), indicate that these enzymes have been largely spread through horizontal gene transfer and are not maintained permanently in the bacterial lineage. For example, not all *Methylocystis*

family of methanotrophs are capable of expressing sMMO (i.e. *Methylocystis* sp. strain M contains a sMMO enzyme, whereas *Mcy. parvus* OBBP does not). The identification of a second lone copy of *mmoX* in *Ms. sporium* and the isolation of plasmids from this organism by previous studies (Lidstrom & Wopat, 1984) gives further information about the evolution of sMMO genes and how some methanotrophs may have acquired these genes through horizontal gene transfer. This also supports the local adaptation hypothesis, which suggest that genes that are important for adaptation to local environment (i.e. sMMO in copper-depleted environments such as peat-bogs) are more likely to be plasmid-encoded than essential 'house-keeping genes' which tend to be chromosomally encoded. None of the sMMO operons sequenced to date have been shown to be plasmid-encoded, suggesting that they have been subjected to long term adaptation in copper-depleted environment and the genes that were probably present on plasmids became mobilised into the chromosome.

The contribution of each copy of *mmoX* in sMMO activity was assessed by constructing double cross-over knock-out mutants. Disruption of *mmoX*1 resulted in an sMMO minus phenotype, whereas disruption to *mmoX*2 had no noticeable affect on sMMO expression. The duplication of *mmoX* was further characterised at the molecular and biochemical level. RT-PCR data obtained suggested that the majority of the *mmoX* transcripts corresponded to *mmoX*1. In comparison, *mmoX*2 transcripts could be detected at relatively low levels. Nevertheless both copies were found to be transcribed. However, following analysis of tryptically digested MmoX polypeptides by means of LC-ESI-MS/MS, no unique peptides corresponding to MmoX2 could be positively identified. It is possible that translation is unable to initiate from the SD sequence of *mmoX*2 due to the presence of overlapping SD sequences, which is affecting the efficiency of translation of the mRNA. Based on translational studies carried out on model organisms such as *E. coli*, it is known that the efficiency of translation of mRNA is affected by the SD sequence and the extent of homology between the SD and 16S rRNA. The efficiency of translation is also affected by the distance between the SD and the translational start codon (Schottel *et al.*, 1984). In light of the data presented in this study, it can be conclude that *mmoX*1 is crucial for sMMO activity, whereas *mmoX*2, which is transcribed only at low levels, is probably not translated, however if it is, it does not play a significant role in sMMO activity. An alternative approach to follow the transcription and translation profile from the σ^{54} promoter of *mmoX*1 and *mmoX*2 would be to construct transcriptional and translation

fusions with one of the suicide promoter probe vectors described in Chapter 4. The activity of the reporter gene could be conveniently assayed, which could be directly correlated to the *in-vivo* transcriptional activity and translation efficiency.

Mutational analysis on *mmoX* gave the first insights into the expression of the water soluble pigment produced by *Ms. sporium*. The pigment was only produced when the sMMO enzyme was expressed and when the bioavailable iron was low since sMMO is an iron-containing enzyme (Elango *et al.*, 1997; Rosenzweig *et al.*, 1993) and under these conditions, iron is rapidly used up. Under iron excess conditions, the pigment was no longer produced. This pigmentation profile could be correlated with iron acquisition mechanisms mediated by siderophores (Schalk *et al.*, 2004). In such systems, siderophores are secreted into the extracellular medium, where they bind iron and are then subsequently transported back into the bacteria. Based on these data, it can be postulated that the water soluble pigment in *Ms. sporium* is operating in a similar fashion as a strategy to acquire iron under iron limiting conditions. However, further work needs to be done to define the exact mechanism of iron acquisition.

The amenability of *Ms. sporium* to genetic manipulations was demonstrated through the heterologous expression and complementation of the sMMO minus mutant strain, *Ms. sporium* Δ*mmoX*1, using the broad-host range plasmids pVK104 and pVK100Sc. All the respective transconjugants selected showed restoration of sMMO activity, however at much reduced activity compared to that of the wild-type strain. Similar reduced activities were observed with identical experiments done on *Mm. album* BG8 and *Mcy. parvus* OBBP with plasmid pVK100Sc containing *Ms. trichosporium* sMMO operon (Lloyd *et al.*, 1999b). In this study, complementation of the sMMO minus mutant of *Ms. sporium*:Δ*mmoX*1 strain with sMMO from both *Ms. trichosporium* and *Mc. capsulatus* was demonstrated. This low level heterologous expression of the sMMO operon is likely to be due to poor recognition of the native promoter and/or poor recognition of the origin of replication of the plasmid in the host. Plasmid incompatibility could also be a factor in low level heterologous expression of sMMO, since there has been a report on *Ms. sporium* containing a plasmid (Lidstrom & Wopat, 1984). However, since the construction of the expression plasmids pVK104 and pVK100Sc, a number of broad-host range plasmids have been used in methanotrophs for the heterologous expression of reporter genes with high activity (Csaki *et al.*, 2003; Theisen *et al.*, 2005).

In order to further improve the heterologous expression of sMMO activity in methanotrophs, as well as in non-methanotrophic strains, new broad-host range plasmids containing the sMMO operon need to be constructed and analysed. With an improved heterologous expression system for sMMO, the full potential of the wide substrate specificity of sMMO can be exploited in biotransformation and bioremediation processes. Through the data presented in this chapter, the amenability of *Ms. sporium* to genetic manipulations has been demonstrated and therefore it could be used as an alternative model organism in the future for investigating the molecular regulation of the expression of MMO enzymes by copper ions.

Chapter 4

Development and Validation of Genetic Tools for Methanotrophs

4.1 Introduction

Since the pioneering work of Whittenbury and co-workers (Whittenbury *et al.*, 1970), the physiology and biochemistry of methanotrophs have been extensively studied (Reviewed in Anthony, 1982). Despite the vast amount of information available on the physiology and biochemistry of methanotrophs, the precise molecular mechanism of the regulation of the expression of methane monooxygenase by copper ions is yet to be elucidated. A contributing factor to this slow progress has been that the development of genetic systems in methanotrophs has been relatively slow. This is partly because of a lack of suitable broad host range plasmids and the absence of effective transformation protocols for methanotrophs. This lack of availability of suitable genetic tools in methanotrophs had greatly hampered earlier works on the detailed molecular studies of the regulation of MMO (Murrell, 1992; Murrell, 1994).

Early attempts to characterise MMO by mutagenesis using various mutagens had mixed success. Mutagenesis with nitrosoguanidine, ethyl methane sulphonate and ultraviolet light gave low frequencies of mutation, however, some success to obtain MMO mutants was achieved using the suicide-substrate dichloromethane (DCM) (McPheat *et al.*, 1987). Several mutants were isolated with DCM that were defective in pMMO and which expressed sMMO constitutively with elevated copper concentrations (Fitch *et al.*, 1993). The major drawbacks of the DCM resistant mutants were that they were very unstable and it was not possible to identify the precise location of the mutation on the chromosome leading to the constitutive nature of sMMO expression.

Since these earlier studies, the availability of improved broad-host range plasmids and the improvements in molecular biology methods have allowed the development of genetic systems in methanotrophs. In addition, gene transfer systems in methanotrophs, such as transformation by electroporation were unreliable and required high concentrations of DNA; however, gene transfer by conjugal transfer proved to be useful (Lidstrom & Stirling, 1990; Murrell, 1992). The development of a protocol for marker-exchange mutagenesis (Martin & Murrell, 1995; Toukdarian & Lidstrom, 1984) for gene knock-outs has been a major break-through in the molecular genetics of methanotrophs and has been the primary strategy for determining the function of key genes. This system is very versatile and since the original mutagenesis, which was carried out on *Ms. trichosporium* (Martin & Murrell, 1995), similar

mutagenesis procedures have been successfully carried out on *Mc. capsulatus* (Csaki *et al.*, 2003) and *Ms. sporium* (Chapter 3).

The construction of *in-vitro* gene fusions with a promoter and a promoterless reporter gene has revolutionised genetic techniques and how they can be applied to analyse gene expression. Gene fusion systems were originally developed in *E. coli* where β-galactosidase, encoded by *lacZ*, was put under the control of new or altered promoter signals and the activity of this enzyme was precisely quantified using a simple and sensitive colorimetric assay (Casadaban *et al.*, 1980). The use of gene fusion technology utilising many different reporter genes has been widely used in bacteria to monitor *in-vivo* gene expression, especially of those genes whose products are difficult to assay (Schweizer & Chuanchuen, 2001). Reporter genes have been used as both transcriptional (operon) and translational (protein) fusions (Silhavy & Beckwith, 1985). In transcriptional fusions, the transcription of a reporter gene is placed under the control of a promoter, where the reporter gene has its own ribosomal binding site. The activity of the reporter gene product is proportional to the level of transcription initiation from the promoter (Linn & Stpierre, 1990). In translational fusions, both the transcription and translation of a reporter gene is placed under the control of a promoter and ribosomal binding site (RBS) and the activity of the reporter gene product can be correlated to the activity of the native gene product that the promoter and RBS normally regulates (Thomas *et al.*, 1982).

The main aim of this study was to develop and establish a set of genetic tools, such as promoter probe vectors, for investigating the molecular regulation of MMO with respect to varying concentrations of copper. This was absolutely necessary despite the advances researchers have made thus far, since only a few broad host range and integrative suicide promoter probe vectors have been tried and tested in methanotrophs up until this study (Csaki *et al.*, 2003; Figueira *et al.*, 2000; Stolyar *et al.*, 2001). One objective was to demonstrate the feasibility of expressing various reporter genes in methanotrophs under the control of the sMMO σ^{54} promoter and to assess their usefulness, in terms of sensitivity and activity, as a selectable marker for the high throughput detection of *mmoXYBZDC* transcription.

In this study, a number of integrative promoter probe vectors, utilising various reporter genes, have been developed for constructing single-copy transcriptional fusions with methanotroph promoters by *in-vitro* manipulation. To demonstrate the

versatility of these promoter probe vectors, *mmoX* σ^{54} promoter from *Mc. capsulatus* was fused to *gfp*, *xylE*, Km^R (kanamycin resistance gene) and *lacZ* and integrated into the chromosome of *Mc. capsulatus*. In addition, low background broad host range promoter probe vectors, utilising *gfp* and *xylE* reporter genes respectively, were constructed. The versatility of these vectors were demonstrated through the construction of separate transcriptional fusion with *gfp* and *mmoX* σ^{54} promoter from *Methylocella silvestris* BL2 (Theisen *et al.*, 2005).

4.2 Construction of integrative promoter probe vectors

4.2.1 Introduction

Plasmids used as a backbone for developing integrative vectors, such as promoter probe vectors and vectors for performing insertional mutagenesis, require several properties for successful integration into the chromosome of recipient. An important property of such vectors is that they have a conditional replicon, such that the plasmid cannot replicate once conjugated into the recipient strain. A positive selection marker, such as antibiotic resistance, is required for the isolation of clones resulting from the integration of the plasmid into the chromosome via recombination between homologous DNA regions. In addition, recognition sites for plasmid mobilisation from a donor to a recipient strain (i.e. most Gram negative bacteria), such as RP4 origin of transfer (*oriT*), is required if conjugation is to be used to introduce the plasmid into the host. *oriT* is the site where DNA cleavage takes place during conjugation and thus allows plasmid transfer (Waterman *et al.*, 1993). In this study, the donor strain *E. coli* S17.1 λ*pir* was used for plasmid mobilisation, since it had all the genes for integrative conjugal transfer functions of the broad-host-range plasmid, RP4, already integrated into the chromosome and thus plasmid transfer occurred by means of biparental mating and no helper plasmid was required (Simon *et al.*, 1983).

The plasmid, pDAH350 obtained from D. A. Hodgson (University of Warwick), was selected for constructing the promoter probe vectors. Plasmid pDAH350 is based on pUC18/19, which are high copy number multiple cloning vectors with a ColE1 origin of replication (*ori*). Origins of replication based on ColE1 have a conditional replicon (suicide plasmids) as they are non-replicative in non-enteric organisms making it ideal for use as a backbone for constructing integrative vectors in methanotrophs. pDAH350 contains a gentamicin resistance (Gm^R) gene on its backbone, which is effective for use as a selectable marker in methanotrophs, and a multiple cloning site (MCS) with a large selection of unique restriction sites, thus facilitating cloning of multiple genes (Figure 4.1).

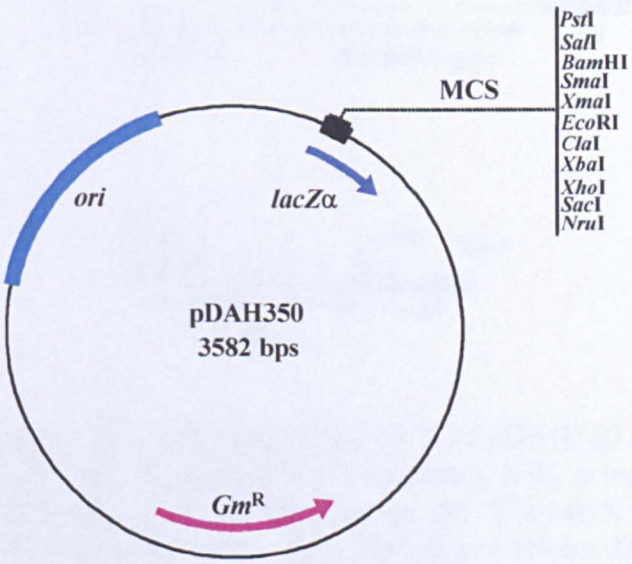


Figure 4.1 Physical map of plasmid pDAH35, obtained from D. A. Hodgson (University of Warwick).

4.2.2 Construction of *gfp* and *xylE* promoter probe vectors

The molecular cloning strategies for constructing a GFP and a XylE promoter probe vector for analysing the *mmoX* σ^{54} promoter activity is described in this section. These promoter probe vectors can then be used to monitor transcription in response to changes in environmental conditions such as copper levels.

Despite plasmid pDAH350 having most of the properties of an integrative vector, it lacked the recognition site for plasmid mobilisation. In order to fulfil the full criteria of an integrative plasmid, an RP4 based *oriT* was cloned into pDAH350. The *oriT* was PCR amplified using primers *oriT*-F (5'-AAG **GAA TTC** GAT GCC GCT TGC-3') and *oriT*-R (5'-AT**C TCG AG**G CTC TCG CCT G-3') from plasmid pMJ153 (obtained from D. A Hodgson, University of Warwick) and cloned into plasmid pDAH350 to yield plasmid pMHA001. The cloning was facilitated by introducing *Eco*RI and *Xho*I restriction sites (highlighted above in bold and also underlined) within the forward and reverse primers, respectively. The initial strategy was to introduce various promoterless reporter genes into pMHA001 via *Sal*I and *Bam*HI sites and a promoter via *Pst*I and *Sal*I sites (Figure 4.2A).

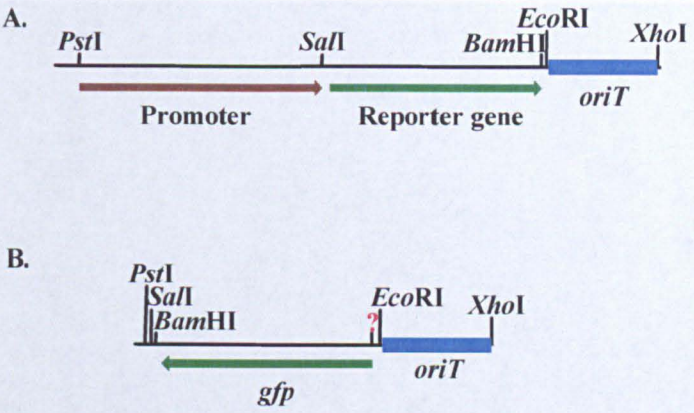


Figure 4.2 Schematic representations of the MCS of pDAH350 containing various genes. A) The proposed MCS of pDAH350 containing *oriT*, a reporter gene, and a promoter sequence using the initial cloning strategy. B) The MCA of pMHA001 with *gfp* inserted in the opposite orientation via a *Bam*HI site (Note: The *Sal*I site is still intact). It is unclear how the 5' end of *gfp* inserted into pMHA001 and therefore is shown by a question mark.

Due to the difficulties in obtaining *E. coli* clones containing plasmid pMHA001 with *gfp*, a colony blot containing 625 *E. coli* clones was probed with *gfp* in an attempt to identify a positive clone (Figure 4.3).

Four clones (clones 172, 253, 529, and 552) hybridised with the *gfp* probe were identified. Restriction digestion analysis revealed that all of the clones, apart from clone 529, contained *gfp*. However, following sequencing of the clones, it was confirmed that the *gfp* was inserted into plasmid pMHA001 via the *Bam*HI site only and also in the opposite orientation (Figure 4.2B). It was unclear how the 5' end of the *gfp* gene had ligated into pMHA001, since the *Sal*I site was still intact.

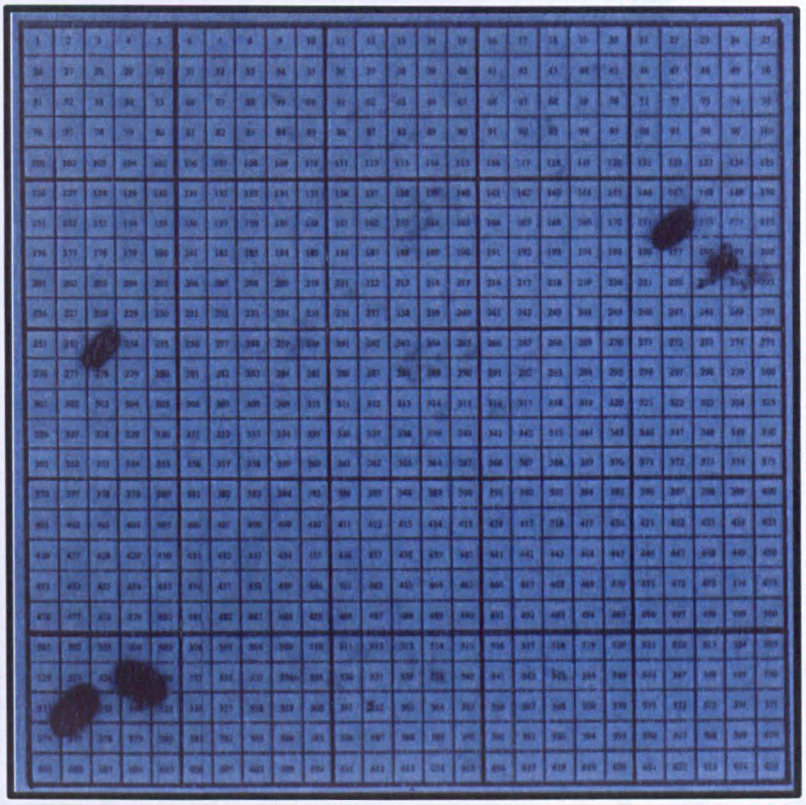


Figure 4.3 Colony blot of 625 *E. coli* clones containing pMHA001, which were hybridised with a *gfp* probe obtained from plasmid pMJ153.

One explanation why *Sal*I enzyme did not cut could be that the *Sal*I and *Bam*HI restriction sites were too close together on the MCS. Therefore, when double digestion was carried out, cleavage with *Bam*HI left only one base pair overhang from the end of the DNA fragment with respect to the *Sal*I recognition site. The number of base pairs from a restriction enzyme recognition site to the end of a DNA fragment can dramatically affect the efficiency of digestion and it was noted that restriction sites for *Sal*I are affected to a greater extent than restriction sites for *Bam*HI (Fermentas Life Sciences, Molecular biology catalogue, 2004-2005).

The above strategy was abandoned due to difficulties in cloning the reporter genes via *Sal*I and *Bam*HI sites. The construction of the integrative promoter probe vectors were pursued via an alternative strategy, which avoided the use of the *Sal*I restriction site (Figure 4.4).

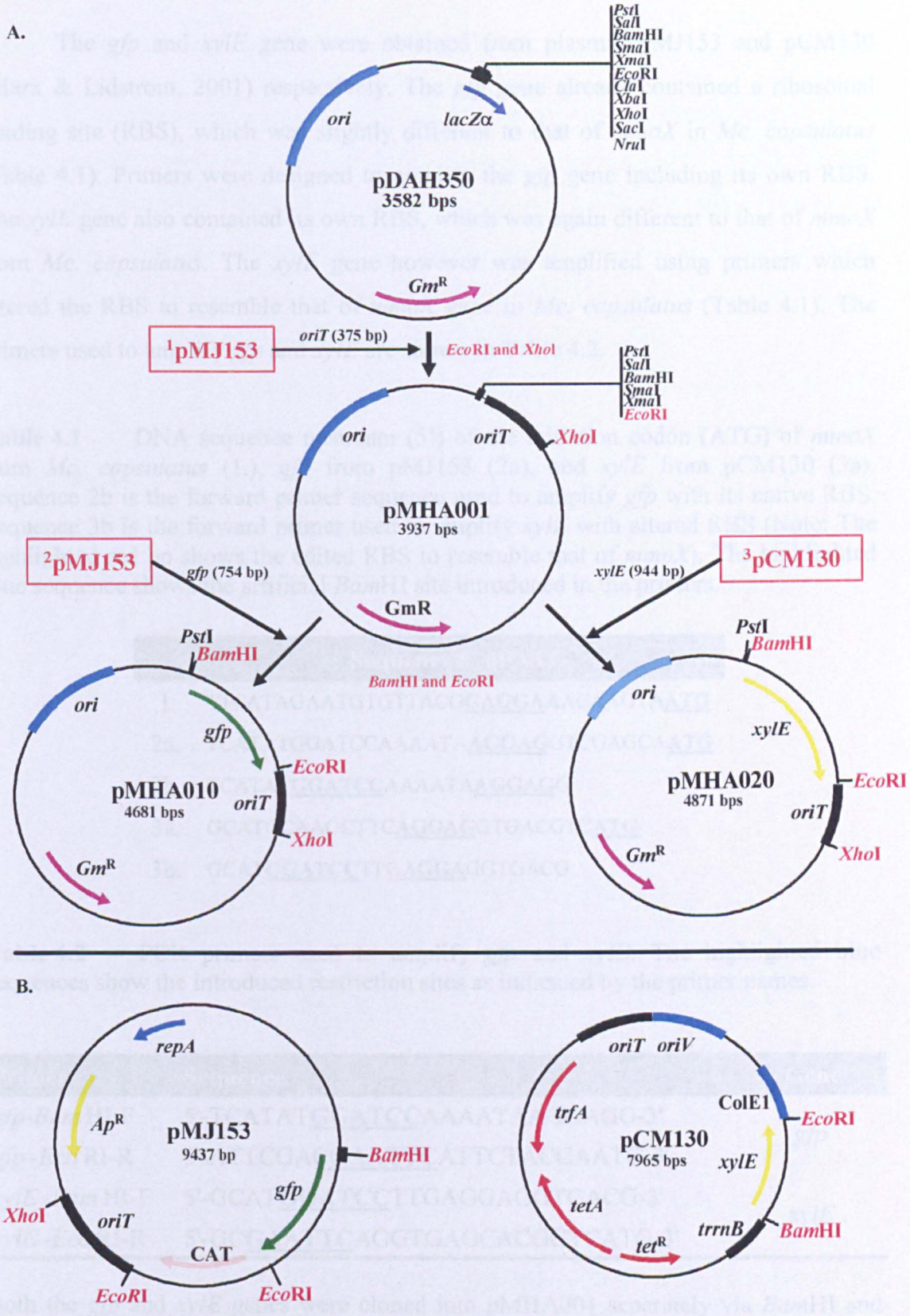


Figure 4.4 A. Schematic representation of the cloning strategy used to construct the promoter probe vectors containing *gfp* (pMHA010) and *xylE* (pMHA020). B. Plasmid pMJ153 was the source of ^{1}*oriT* (375 bp) and ^{2}*gfp* (754 bp). Plasmid pCM130 was the source of ^{3}*xylE* (944 bp). The restriction sites highlighted in red are those introduced through PCR to facilitate cloning.

The *gfp* and *xylE* gene were obtained from plasmid pMJ153 and pCM130 (Marx & Lidstrom, 2001) respectively. The *gfp* gene already contained a ribosomal binding site (RBS), which was slightly different to that of *mmoX* in *Mc. capsulatus* (Table 4.1). Primers were designed to amplify the *gfp* gene including its own RBS. The *xylE* gene also contained its own RBS, which was again different to that of *mmoX* from *Mc. capsulatus*. The *xylE* gene however was amplified using primers which altered the RBS to resemble that of *mmoX* gene in *Mc. capsulatus* (Table 4.1). The primers used to amplify *gfp* and *xylE* are shown in Table 4.2.

Table 4.1 DNA sequence upstream (5') of the initiation codon (ATG) of *mmoX* from *Mc. capsulatus* (1.), *gfp* from pMJ153 (2a), and *xylE* from pCM130 (3a). Sequence 2b is the forward primer sequence used to amplify *gfp* with its native RBS. Sequence 3b is the forward primer used to amplify *xylE* with altered RBS (Note: The highlighted red bp shows the edited RBS to resemble that of *mmoX*). The highlighted blue sequence shows the artificial *Bam*HI site introduced in the primers.

	Sequence 5' of ATG
1	TTCATAGAATGTGTTACG**GAGGA**AACAAGTA**ATG**
2a.	TCATATGGATCCAAAATA**AGGAG**GTCGAGCA**ATG**
2b.	TCATAT**GGATCC**AAAATA**AGGAG**G
3a.	GCATGCAAGCTTC**AGGAG**GTGACGTC**ATG**
3b.	GCAT**GGATCC**TT**G****AGGA**GGTGACG

Table 4.2 PCR primers used to amplify *gfp* and *xylE*. The highlighted blue sequences show the introduced restriction sites as indicated by the primer names.

Primer	Sequence	Gene
gfp-Bam HI-F	5'-TCATAT**GGATCC**AAAATAAGGAGG-3'	*gfp*
gfp -Eco RI-R	5'-ATTCGAC**GAATTC**ATTCTACGAATG-3'	
xylE -Bam HI-F	5'-GCAT**GGATCC**TTGAGGAGGTGACG-3'	*xylE*
xylE -Eco RI-R	5'-GC**GAATTC**AGGTGAGCACGGTCATG-3'	

Both the *gfp* and *xylE* genes were cloned into pMHA001 separately via *Bam*HI and *Eco*RI sites, which were introduced through PCR, to give the general promoter probe vectors pMHA010 and pMHA020 respectively (Figure 4.4).

4.2.3 *In-vitro gfp* and *xylE* transcriptional fusions

To determine the ability of pMHA010 and pMHA020 to monitor transcription and to express functional GFP and XylE in methanotrophs, *in-vitro* transcriptional fusions with *mmoX* σ^{54} promoter from *Mc. capsulatus* were constructed. The *mmoX* σ^{54} promoter from *Mc. capsulatus* was chosen because the promoter region has been mapped previously and its transcriptional activity had been assayed rigorously under varying copper conditions (Csaki *et al.*, 2003; Nielsen *et al.*, 1996).

The *mmoX* σ^{54} promoter without the RBS was PCR amplified from *Mc. capsulatus* using primers *mmoX*-σ^{54}-F (5'-CGC GTT GAT CAG GCT GGT CTT G-3') and *mmoX*-σ^{54}-R (5'-TTG GAT CCA TGA TGA ATG CCC GAT GA-3') and cloned into pMHA010 and pMHA020 via *Pst*I and *Bam*HI sites to give the constructs pMHA011 and pMHA021 respectively (Figure 4.5).

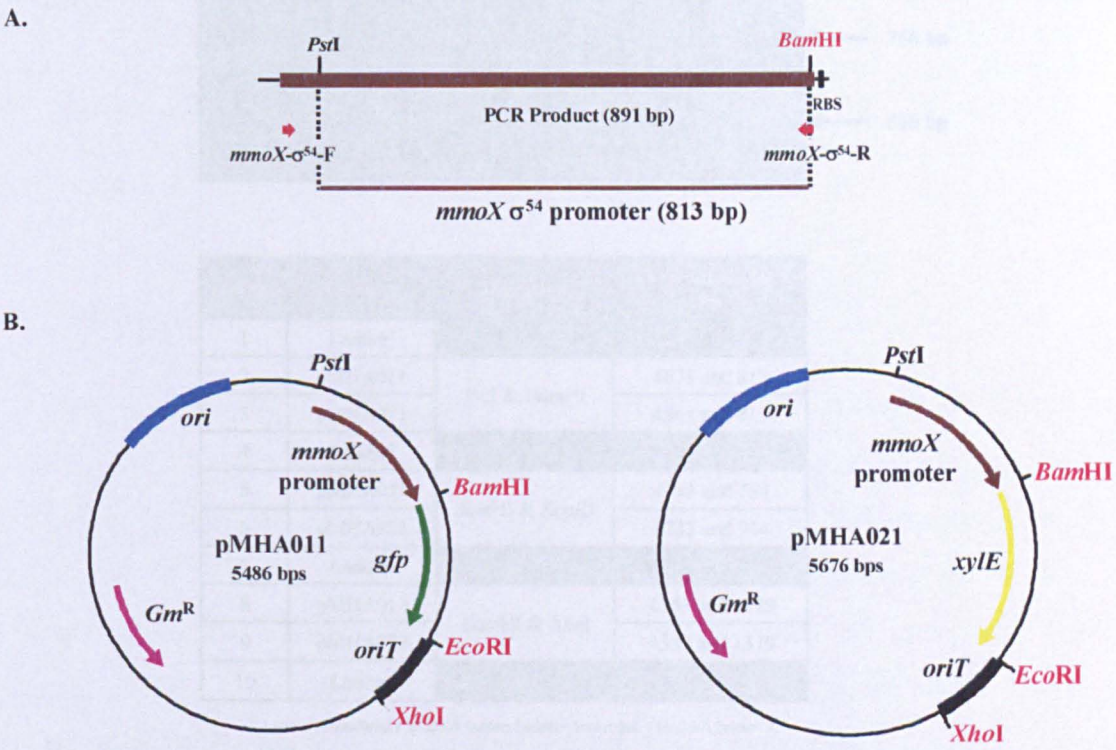
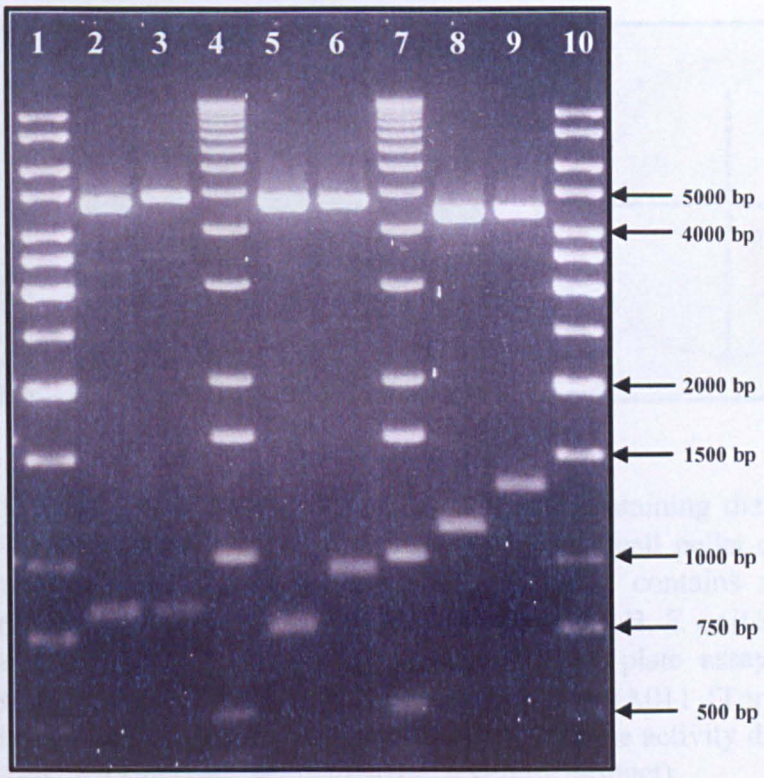


Figure 4.5 Physical map of *gfp* and *xylE* promoter probe vector fused to *mmoX* σ^{54} promoter. The *mmoX* σ^{54} promoter region from *Mc. capsulatus* (A). The σ^{54} promoter region cloned into pMHA010 and pMHA020 are shown between the dotted lines, which does not include the RBS. (Note: No *Pst*I site was introduced within the forward primer since there was a *Pst*I site within the 5' region of the PCR product; however a *Bam*HI site was introduced via the reverse primer to facilitate cloning). (B). A schematic representation of constructs pMHA011 and pMHA021. The restriction sites highlighted in red are those introduced via PCR to facilitate cloning.

The construction of pMHA011 and pMHA021 was confirmed by restriction digest analysis using various combinations of enzymes (Figure 4.6).



Lane	Content	Digestion	Fragment size (bp)
1	Ladder[1]		
2	pMHA011	*Pst*I & *Bam*HI	4673 and 813
3	pMHA021		4863 and 813
4	Ladder[2]		
5	pMHA011	*Bam*HI & *Eco*RI	4732 and 754
6	pMHA021		4732 and 944
7	Ladder[2]		
8	pMHA011	*Bam*HI & *Xho*I	4357 and 1129
9	pMHA021		4357 and 1319
10	Ladder[1]		

Ladder[1]: GeneRuler 1 kb DNA ladder; Ladder[2]: Invitrogen 1 kb DNA ladder

Figure 4.6 Confirmation of the construction of the promoter probe vectors, pMHA011 and pMHA021. Agarose gel (1 % w/v) ran in 1 x TAE buffer containing various restriction digests of DNA from plasmids pMHA011 and pMHA021. The table shows the contents of each lane and the lengths of the expected DNA fragments from the digestions.

The construction of plasmid pMHA011 and pMHA021 was also confirmed through the observation of GFP and XylE activity in whole cells (Figure 4.7).

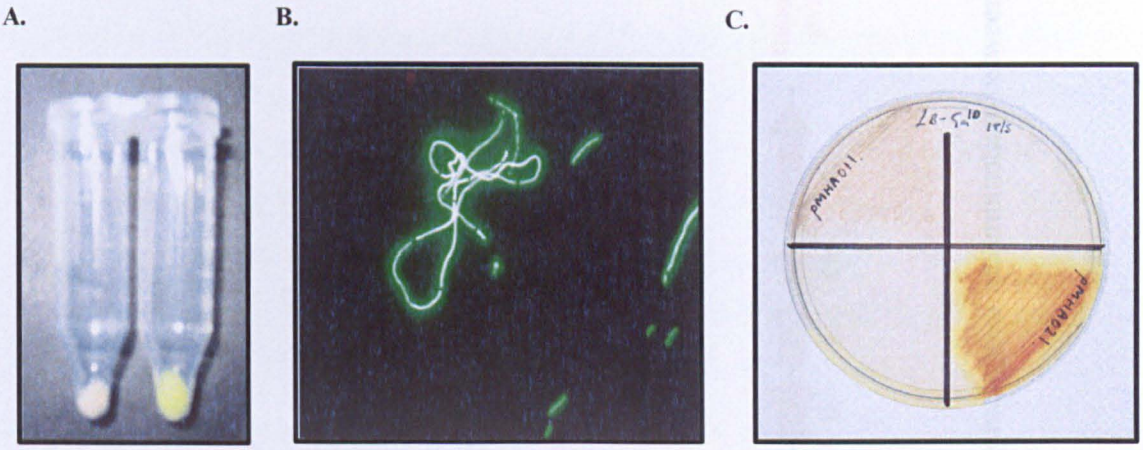


Figure 4.7 GFP and XylE activity in *E. coli* S17.1 λ*pir* containing the promoter probe vector pMHA011 and pMHA021. A. *E. coli* S17.1 λ*pir* cell pellet containing pMHA021 (left tube – No fluorescence since the construct contains *xylE*) and pMHA011 (right tube – green tinge depicting GFP fluorescence). B. *E. coli* S17.1 λ*pir* culture viewed under a fluorescent microscope. C. Catechol plate assay (Section 2.10.3) performed on *E. coli* S17.1 λ*pir* cells containing pMHA011 (Top-left - no activity) and pMHA021 (bottom right – catechol 2, 3-dioxygenase activity detected by the production of 2-hydroxymuconic semialdehyde, a yellow product).

4.2.4 GFP and XylE reporter strains of *Mc. capsulatus*

The newly constructed promoter probe vectors, pMHA011 and pMHA021, containing the *mmoX* σ^{54} promoter fused to *gfp* and *xylE* were integrated into the chromosome of *Mc. capsulatus* by conjugation using the biparental mating method described in Section 2.5.1. The vectors integrated into the chromosome of *Mc. capsulatus* by single homologous recombination via the *mmoX* σ^{54} promoter. The general genotypes of the respective reporter strains are shown schematically in Figure 4.8. Transconjugants and thus the reporter strains were isolated through selection on NMS agar plates containing gentamicin (5 μg ml^{-1}).

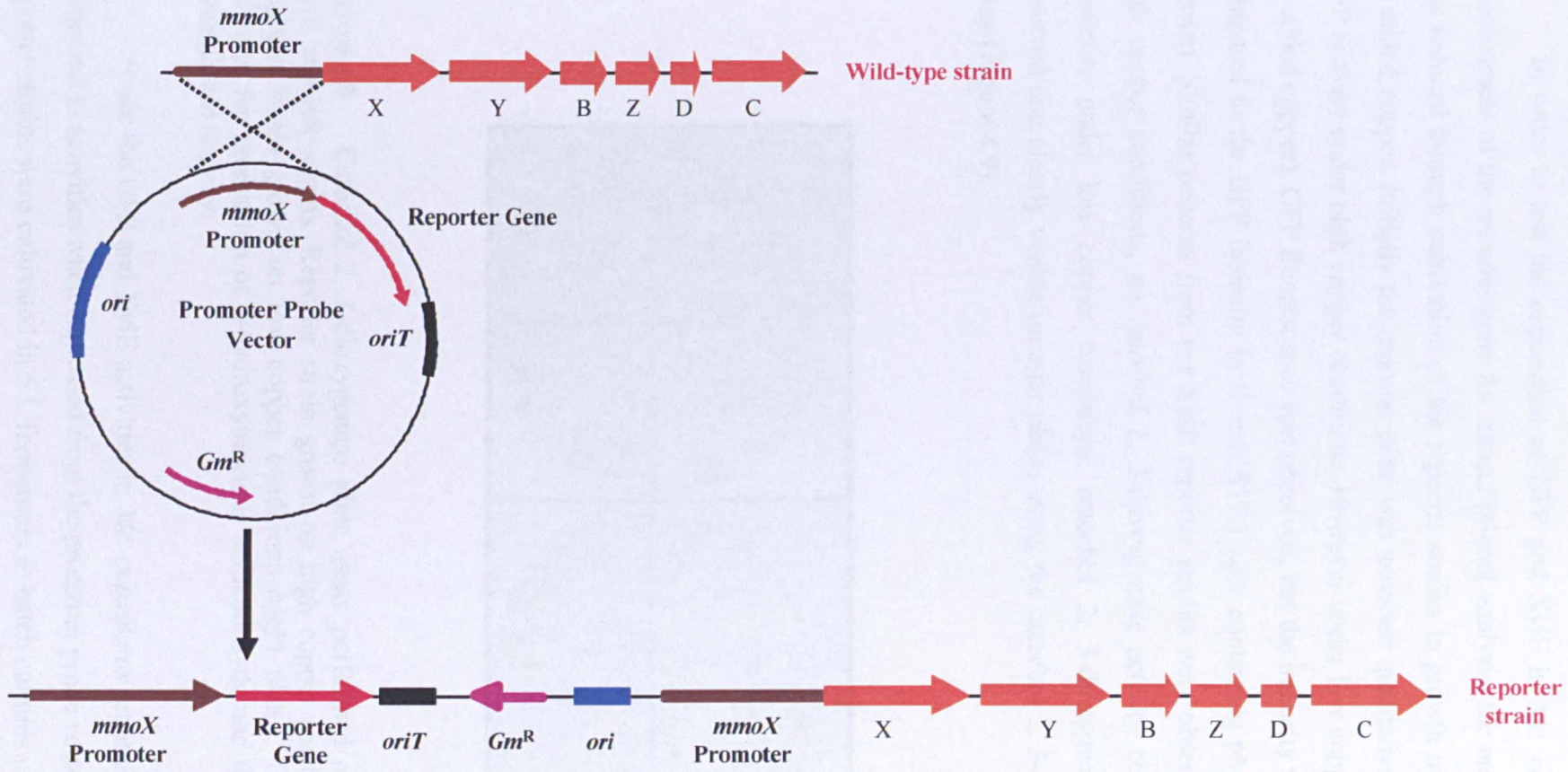


Figure 4.8 Schematic representation of the genotype of a reporter strain following single homologous recombination between the *mmoX* σ^{54} promoter in the promoter probe vector and the chromosome of *Mc. capsulatus*.

4.2.5 Activity of GFP and XylE in *Mc. capsulatus*

In order to test the expression of GFP and XylE in *Mc. capsulatus* and the effectiveness of the reporter gene for transcriptional analysis, the *mmoX* σ^{54} promoter was induced through cultivation of the reporter strains in growth medium containing no added copper. Initially the reporter gene was assessed qualitatively. There was no GFP activity under high copper conditions. However under low copper conditions (i.e no added copper), GFP fluorescence was observed, but the intensity was relatively low compared to the GFP intensity in *E. coli* S17.1 λ*pir* containing pMHA011 (data not shown). Similar patterns from the XylE reporter strains were observed, where under high copper conditions, no catechol 2, 3-dioxygenase activity could be observed. However under low copper conditions, catechol 2, 3-dioxygenase activity was observed and clearly visible on agar plates using the catechol 2, 3-dioxygenase plate assay (Figure 4.9).

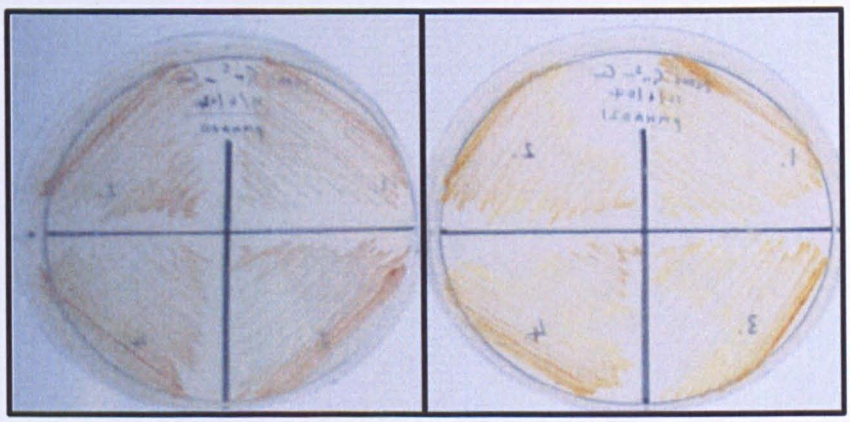


Figure 4.9 Catechol 2, 3-dioxygenase plate assay performed on *Mc. capsulatus* XylE reporter strains. Reporter strain grown on high copper conditions (left plate). Reporter strain grown on low copper conditions (right plate). The yellow colour indicates the production of 2-hydroxymuconic semialdehyde and thus catechol 2, 3-dioxygenase activity.

Since the GFP and XylE activities in *Mc. capsulatus* reporter strains were low compared to activities when expressed from the promoter probe vectors in *E. coli*, both reporter strains were cultivated in 5 L fermentors in batch cultures under high and low copper conditions (Figure 4.10). Since the intracellular location of MMO activity is

dependent on the copper-to-biomass ratio, cultivation of the reporter strains in a 5 L fermentor allowed an easily observable "copper-switch" from pMMO expression to sMMO expression.

Mc. capsulatus cells expressing pMMO and sMMO were harvested during exponential growth (OD_{540} 2-6) and cell-free extracts were prepared as described in Section 2.9.1 (Note: sMMO expressing cells from XylE reporter strain were obtained from early exponential phase (Batch 1) and mid-exponential growth phase (Batch 2)). *Mc. capsulatus* wild-type strain was used as a negative control. The detection of sMMO activity under low copper-to-biomass ratio using the naphthalene assay indicated that the integration of the promoter probe vector upstream of the *mmoX* σ^{54} promoter did not create a detectable polar affect on the transcription and thus did not affect the expression of the sMMO genes. The specific activities of GFP and XylE were quantified from the cell-extracts described above using methods described in Sections 2.10.2 and 2.10.3 respectively (Figure 4.11).

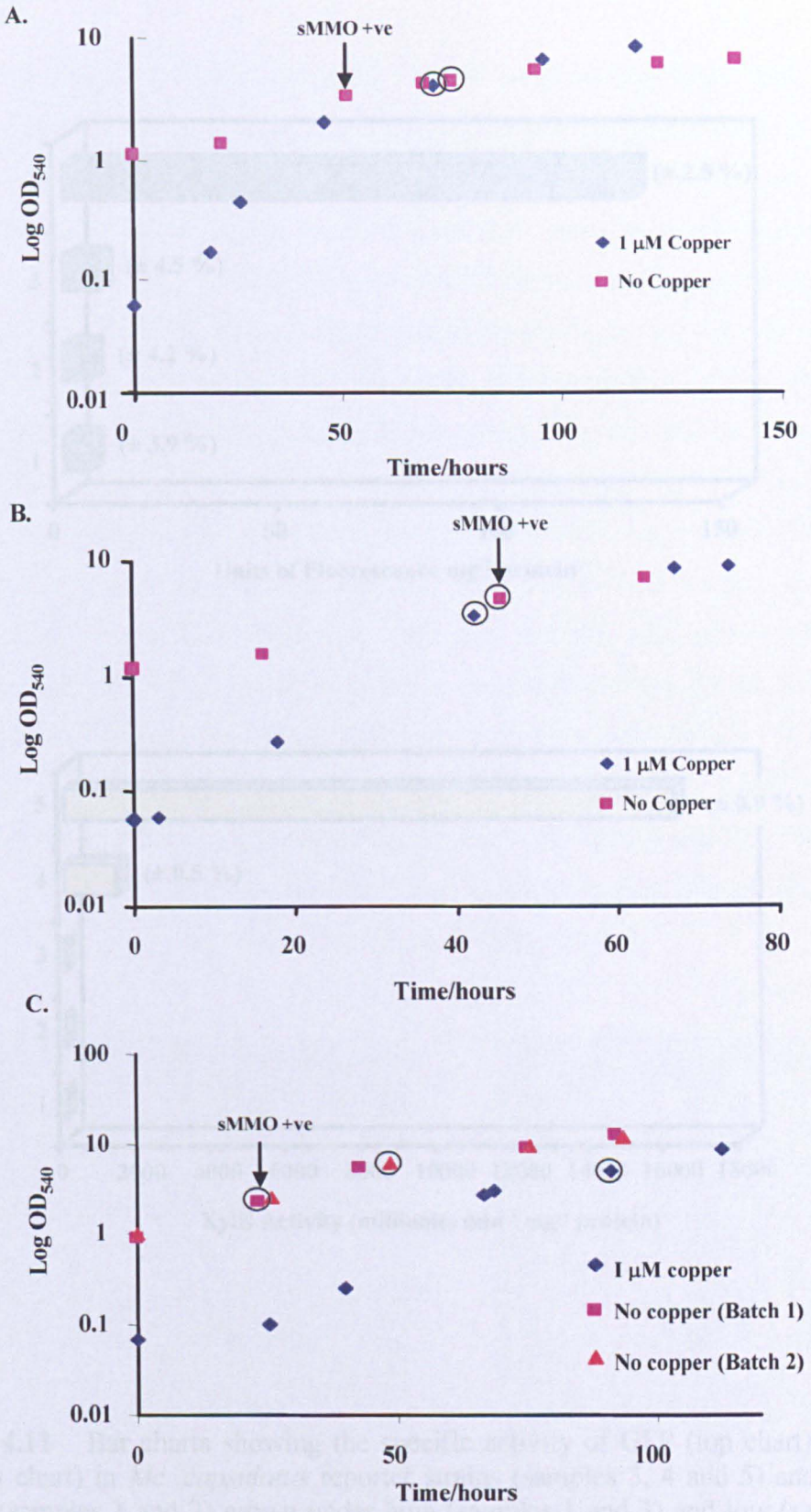


Figure 4.10 Growth curves of *Mc. capsulatus* wild-type strain (A.), GFP reporter strain (B.), and XylE reporter strain (C.) cultivated in a 5 L fermentor. All strains were initially cultivated under high copper conditions (1 µM copper) and then under low copper (no added copper) conditions. The XylE reporter strain was cultivated for a second time with no added copper to the medium (labelled as batch 2). The initial detection of sMMO activity, using the naphthalene assay, is indicated. The points at which cells were removed to prepare cell-free extracts are also indicated by circles.

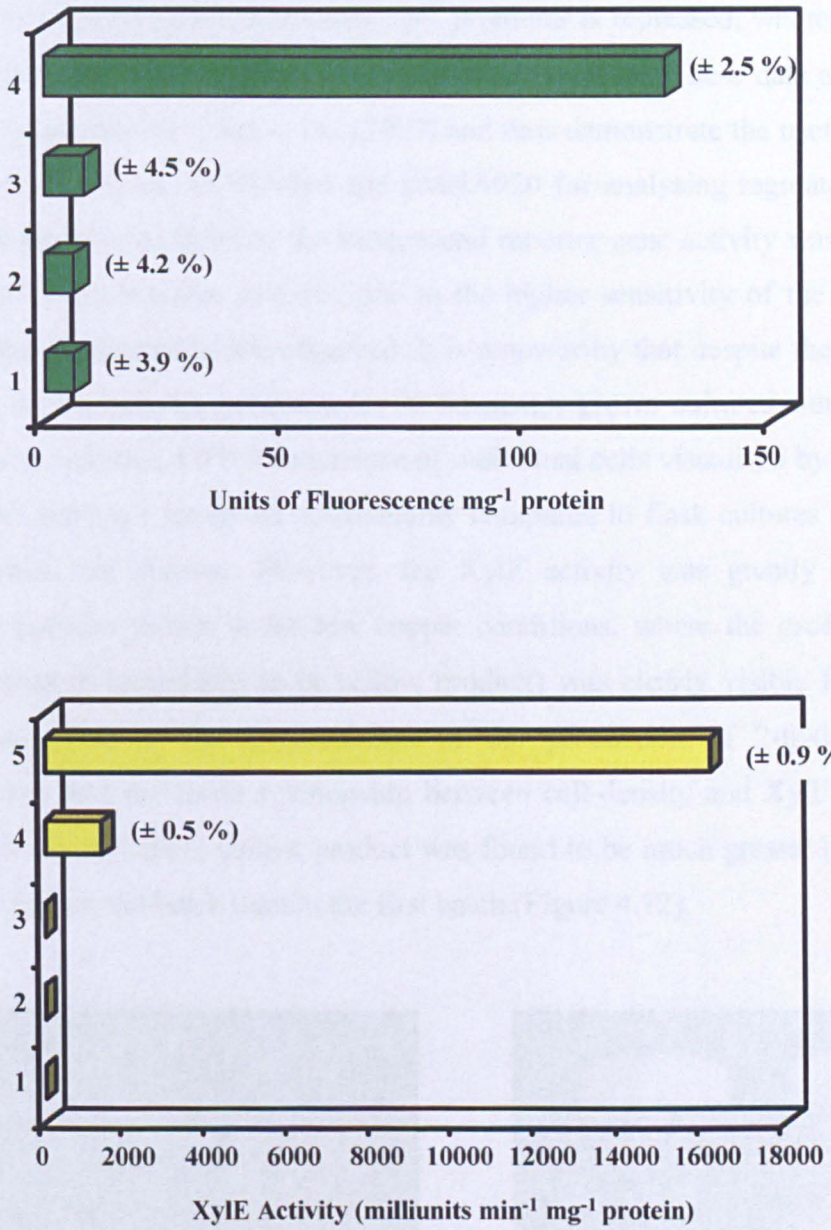


Figure 4.11 Bar charts showing the specific activity of GFP (top chart) and XylE (bottom chart) in *Mc. capsulatus* reporter strains (samples 3, 4 and 5) and wild-type strains (samples 1 and 2) grown under high (samples 1 and 3) and low (samples 2, 4 and 5) copper conditions. (Note: Samples 4 and 5 in the XylE bar chart represent the activity from batch 1 and 2 respectively, see Figure 4.10). GFP activity represents a mean of two independent assays and XylE activity represents a mean of ΔOD_{375} min^{-1} over at least 2 minutes. The mean standard deviations (expressed as percentage) are shown in brackets.

The data presented in Figure 4.11 clearly indicated that under high copper-to-biomass ratio, transcription from *mmoX* σ^{54} promoter is repressed, whereas under low copper-to-biomass ratio, transcription is highly upregulated. These data are consistant with those presented by Csaki *et al.*, (2003) and thus demonstrate the usefulness of the promoter probe vectors pMHA010 and pMHA020 for analysing regulatory elements such as promoters. In addition, the background reporter gene activity was very low in the case of XylE, whereas in GFP, due to the higher sensitivity of the fluorometer, slight background activity was observed. It is noteworthy that despite the massive up-regulation of the reporter gene activity in fermentor grown cultures cultivated under low copper conditions, GFP fluorescence of individual cells visualised by fluorescence microscopy were not improved considerably compared to flask cultures or agar plate cultures (data not shown). However, the XylE activity was greatly enhanced in fermentor cultures grown under low copper conditions, where the production of 2-hydroxymuconic semialdehyde (a yellow product) was clearly visible following the XylE assay. Due to the accumulation of the production of 2-hydroxymuconic semialdehyde and the linear relationship between cell-density and XylE activity, the intensity of the diffusible yellow product was found to be much greater (i.e. 11.5 fold higher) in the second batch than in the first batch (Figure 4.12).

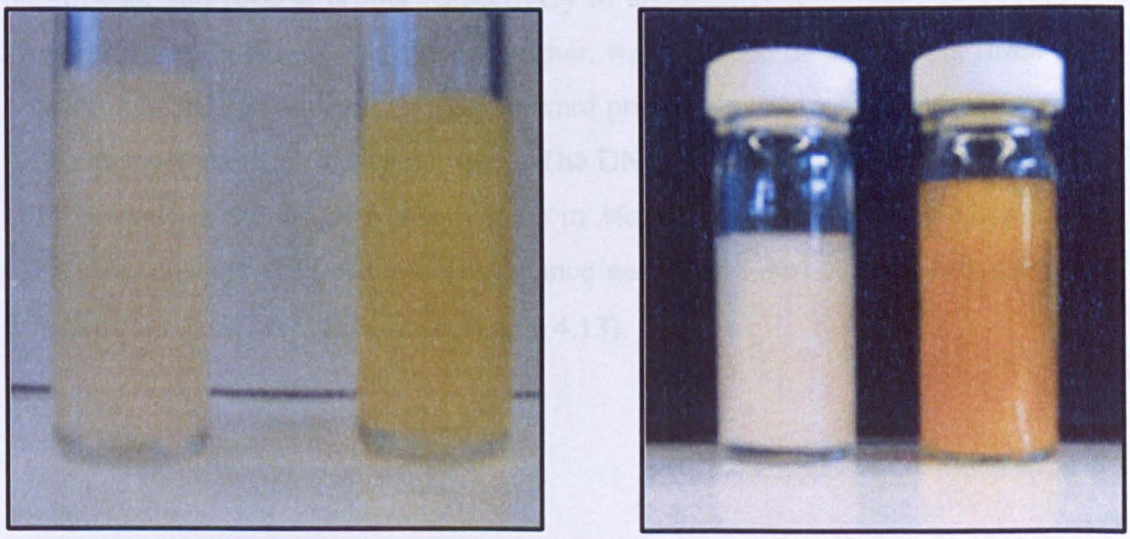


Figure 4.12 Qualitative XylE assay. Cultures of *Mc. capsulatus* XylE reporter strain expressing sMMO from batch 1 and batch 2 with saturated amounts of catechol and no added catechol. Note: The intensity of the diffusible yellow product is proportional to the XylE activity.

To get an overview on the relative expression of the reporter gene following single copy integration into the chromosome compared to the expression level from a multi-copy plasmid, *E. coli* S17.1 λ*pir* strains containing pMHA011 and pMHA021 were cultivated. Cell-free extracts were prepared from exponentially growing flasks cultures (OD_{600} 0.4-0.8) and the GFP and XylE activity was quantified. The GFP activity in *E. coli* was found to be approximately 21 fold higher than in the reporter strain, whereas the XylE activities were found to be 17 (Batch 1) and 1.5 (Batch 2) fold higher in *E. coli* than in the reporter strain.

4.3 Construction of a kanamycin resistance promoter probe vector

The construction of integrative vectors and the principal uses of promoter probe vectors have been demonstrated in the previous section using both GFP and XylE activity as a marker to follow the transcription initiation from the *mmoX* σ^{54} promoter in *Mc. capsulatus*. In this section, the construction of an additional promoter probe vector containing a kanamycin resistance gene as a reporter is described.

The kanamycin resistance gene from the multiple cloning vector, pK18mob, was PCR amplified using primers *Km*R-F and *Km*R-R, which gave an 842 bp PCR product. These primers were designed with a *Bam*HI and *Eco*RI restriction site within the forward and reverse primer respectively to facilitate cloning into pMHA011. The *Bam*HI restriction site, on the forward primer, was included on a DNA tail fused to the 5' portion of the forward primer. The forward primer started from the initiation codon, ATG, of the kanamycin resistance gene. The DNA tail was comprised of the 34 bp of the 5' region of the ATG of *mmoX* gene in *Mc. capsulatus*, which included its own RBS. This ensured the kanamycin resistance gene was fused exactly with the *mmoX* σ^{54} promoter from *Mc. capsulatus* (Figure 4.13).

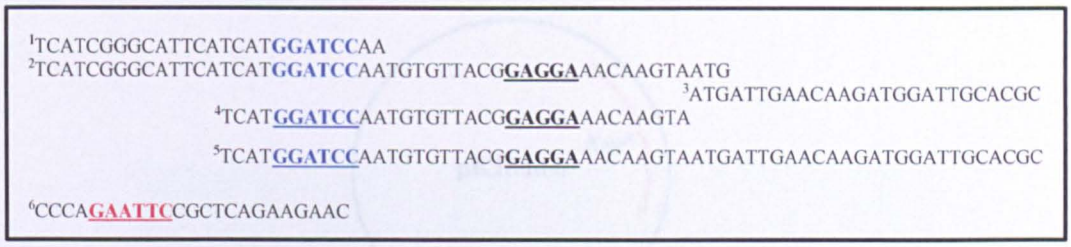


Figure 4.13 Primer sequences of the DNA tail fused to *Km*R-F primer for the construction of an exact transcriptional fusion with *mmoX* σ^{54} promoter from *Mc. capsulatus*. [1]Reverse complement of *mmoX*-σ^{54}-R primer (See Section 4.2.3). [2]*mmoX*-σ^{54}-R primer sequence plus the downstream DNA sequence leading to the ATG of *mmoX*. [3]KmR-F primer used to amplify kanamycin resistance gene without the DNA tail. [4]DNA tail. [5]*Km*R-F primer including the DNA tail. [6]*Km*R-R primer used to amplify kanamycin resistance gene. The sequence highlighted in blue shows the introduced *Bam*HI site and the highlighted red sequence shows the introduced *Eco*RI site. The RBS is highlighted in bold.

The *gfp* gene from pMHA011 (Figure 4.5) was removed following *Bam*HI and *Eco*RI digestion and the kanamycin resistance gene was ligated following digestion using the same restriction enzymes to give the new kanamycin promoter probe vector, pMHA035 (Figure 4.14).

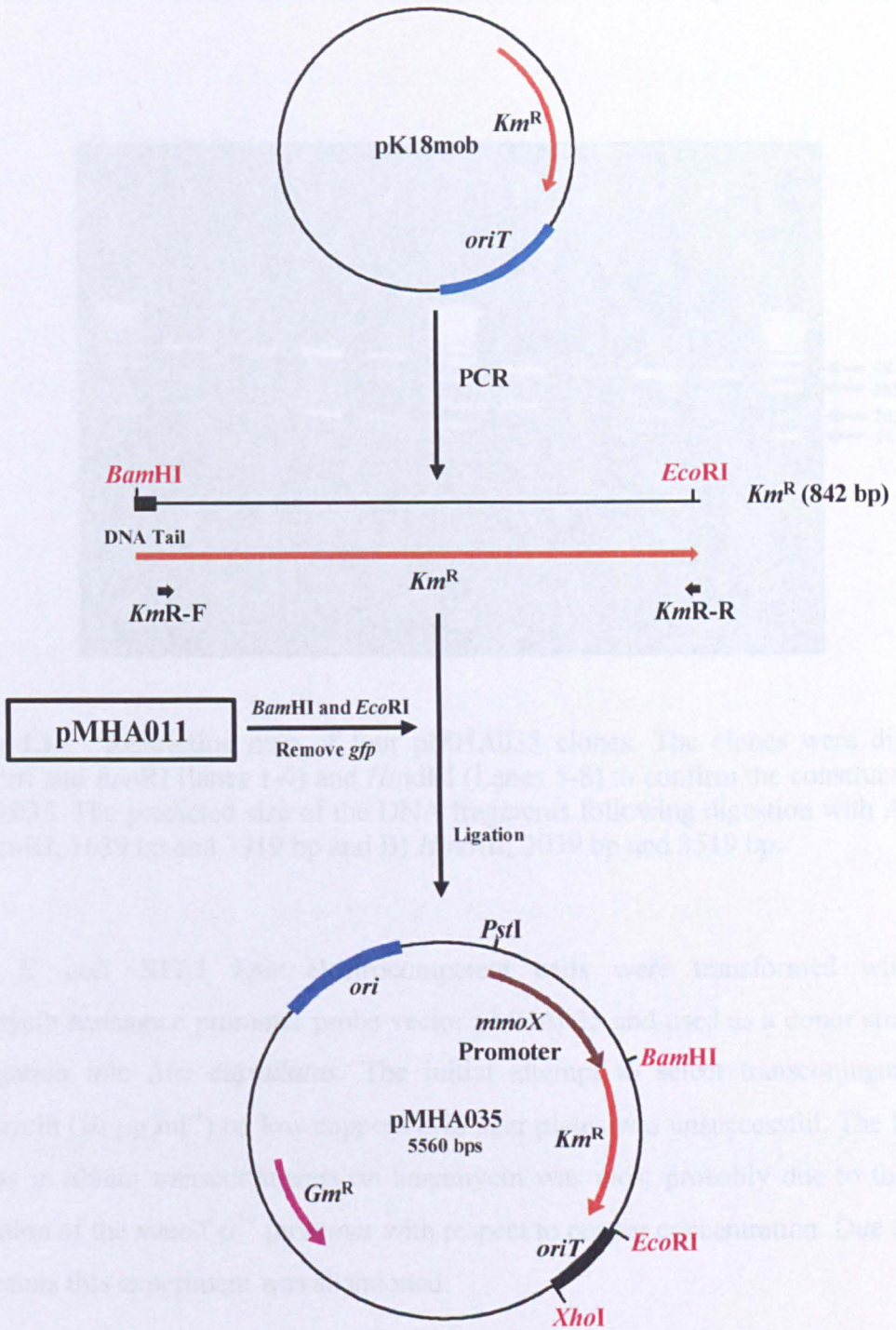


Figure 4.14 The cloning strategy for the construction of the kanamycin resistance promoter probe vector, pMHA035. The restriction sites highlighted in red are those introduced through PCR to facilitate cloning.

The construction of pMHA035 was confirmed by restriction digest analysis (Figure 4.15).

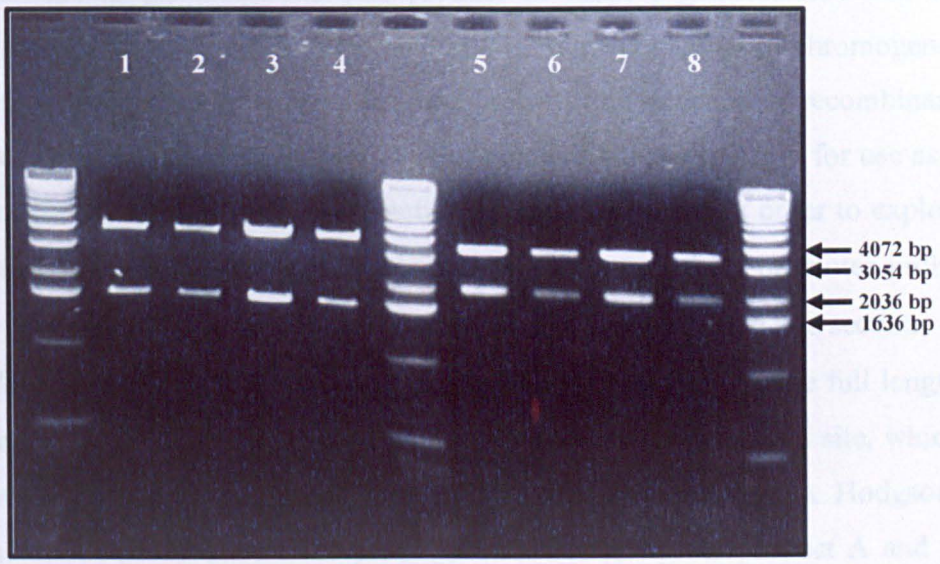


Figure 4.15 Restriction map of four pMHA035 clones. The clones were digested with *Pst*I and *Eco*RI (lanes 1-4) and *Hin*dIII (Lanes 5-8) to confirm the construction of pMHA035. The predicted size of the DNA fragments following digestion with A) *Pst*I and *Eco*RI; 1639 bp and 3919 bp and B) *Hin*dIII; 2039 bp and 3519 bp.

E. coli S17.1 *λpir* electrocompetent cells were transformed with the kanamycin resistance promoter probe vector pMHA035 and used as a donor strain for conjugation into *Mc. capsulatus*. The initial attempt to select transconjugants on kanamycin (10 μg ml^{-1}) on low copper NMS agar plates was unsuccessful. The lack of success in obtain transconjugants on kanamycin was most probably due to the tight regulation of the *mmoX* σ^{54} promoter with respect to copper concentration. Due to time constraints this experiment was abandoned.

4.4 Construction of a LacZ promoter probe vector

Promoter probe vectors utilising *lacZ* as a reporter gene has been most widely used for constructing gene fusions. The product of *lacZ*, β-galactosidase can be relatively easily quantified and due to the availability of large number of chromogenic and fluorogenic substrates, it is widely used in *E. coli* for the selection of recombinant clones. One of the aims of this study was to assess different reporter genes for use as a genetic screen for the high throughput detection of MMO mutants. In order to exploit the usefulness of β-galactosidase activity in *Mc. capsulatus*, a *lacZ* promoter probe vector fused to *mmoX* σ^{54} promoter was construction and is described in this section.

The *lacZ* gene is 3,075 bp and several attempts to PCR amplify the full length gene were unsuccessful. Fortunately within the *lacZ* gene there was a *Sac*I site, which allowed the amplification of *lacZ* gene from pDAH274 (obtained from D. A. Hodgson) in two segments. The respective PCR products were designated as product A and B from the *lacZ* promoter probe vector, pDAH274 (Figure 4.16). Similar to the amplification of the kanamycin resistance gene in pMHA035, the *lacZ* gene was amplified with a DNA tail, which was fused to the 5' portion of the forward primer of product A in order to construct an exact transcriptional fusion between *lacZ* and *mmoX* σ^{54} promoter. The primers used to amplify *lacZ* are shown in Table 4.3.

Table 4.3 PCR primers used to amplify *lacZ* from pDAH274. The sequence highlighted in blue indicates the location of a *Bam*HI site and the red sequence indicates an *Eco*RI restriction site.

Primers	Sequence
lacZ-AF	TCATGGATCCAATGTGTTACGGAGGAAACAAGTAATGACCATGATTACGGATTCACTGG
lacZ-AR	CCAGTGCAGGAGCTCGTTATC
lacZ-BF	CGATAACGAGCTCCTGCACTG
lacZ-BR	CTTACGCGAATTCCGGGCAGAC

In order to confirm the attachment of the DNA tail containing the *Bam*HI site on the forward primer of product A and the nucleotide sequence of the *lacZ* gene, both product A and B was cloned into pCR2.1-TOPO cloning vector (Invitrogen) and sequenced using M13 universal primers.

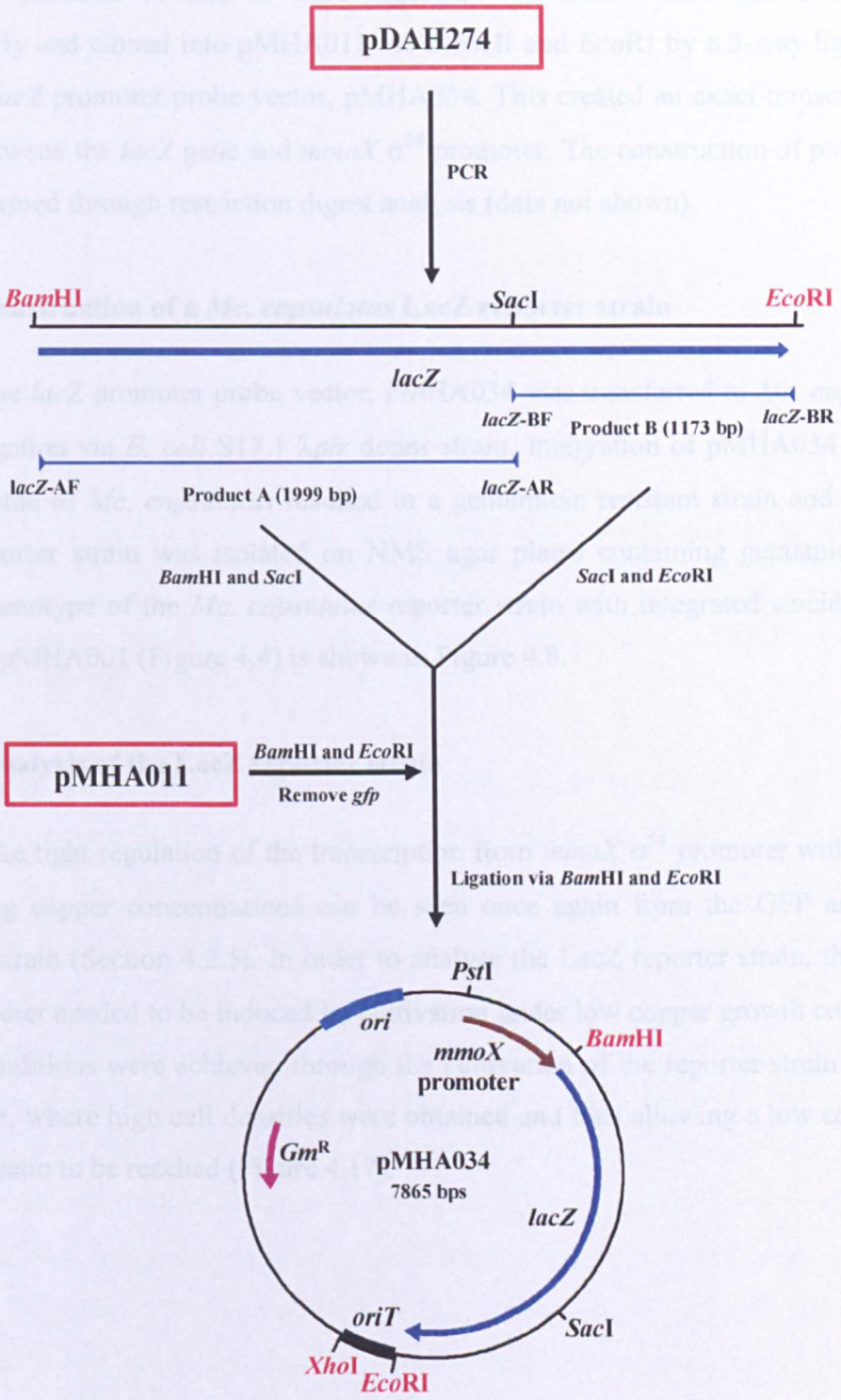


Figure 4.16 The cloning strategy for the construction of the *lacZ* promoter probe vector, pMHA034. The restriction sites highlighted in red are those introduced through PCR to facilitate cloning.

The *lacZ* products A and B were digested with *Bam*HI-*Sac*I and *Sac*I-*Eco*RI respectively and cloned into pMHA011 via *Bam*HI and *Eco*RI by a 3-way ligation to give the *lacZ* promoter probe vector, pMHA034. This created an exact transcriptional fusion between the *lacZ* gene and *mmoX* σ^{54} promoter. The construction of pMHA034 was confirmed through restriction digest analysis (data not shown).

4.4.1 Construction of a *Mc. capsulatus* LacZ reporter strain

The *lacZ* promoter probe vector, pMHA034 was transferred to *Mc. capsulatus* by conjugation via *E. coli* S17.1 λ*pir* donor strain. Integration of pMHA034 into the chromosome of *Mc. capsulatus* resulted in a gentamicin resistant strain and thus the LacZ reporter strain was isolated on NMS agar plates containing gentamicin. The general genotype of the *Mc. capsulatus* reporter strain with integrated suicide vector based on pMHA001 (Figure 4.4) is shown in Figure 4.8.

4.4.2 Analysis of the LacZ reporter strain

The tight regulation of the transcription from *mmoX* σ^{54} promoter with respect to varying copper concentrations can be seen once again from the GFP and XylE reporter strain (Section 4.2.5). In order to analyse the LacZ reporter strain, the *mmoX* σ^{54} promoter needed to be induced by cultivation under low copper growth conditions. These conditions were achieved through the cultivation of the reporter strain in a 5 L fermentor, where high cell densities were obtained and thus allowing a low copper-to-biomass ratio to be reached (Figure 4.17).

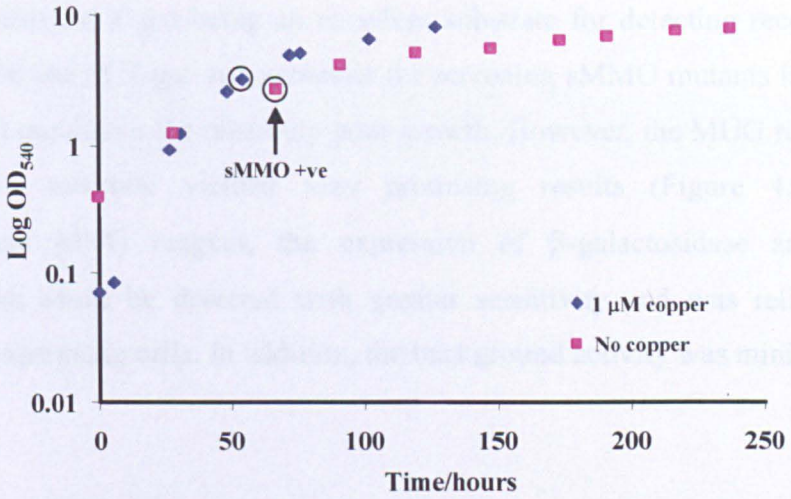


Figure 4.17 Growth curves of *Mc. capsulatus* LacZ reporter strain. The strain was initially cultivated under high copper conditions (1 µM copper) and then under low copper (No added copper) conditions. The initial detection of sMMO activity using the naphthalene assay is indicated. The points at which cells were removed for analysis of β-galactosidase activity are indicated by circles.

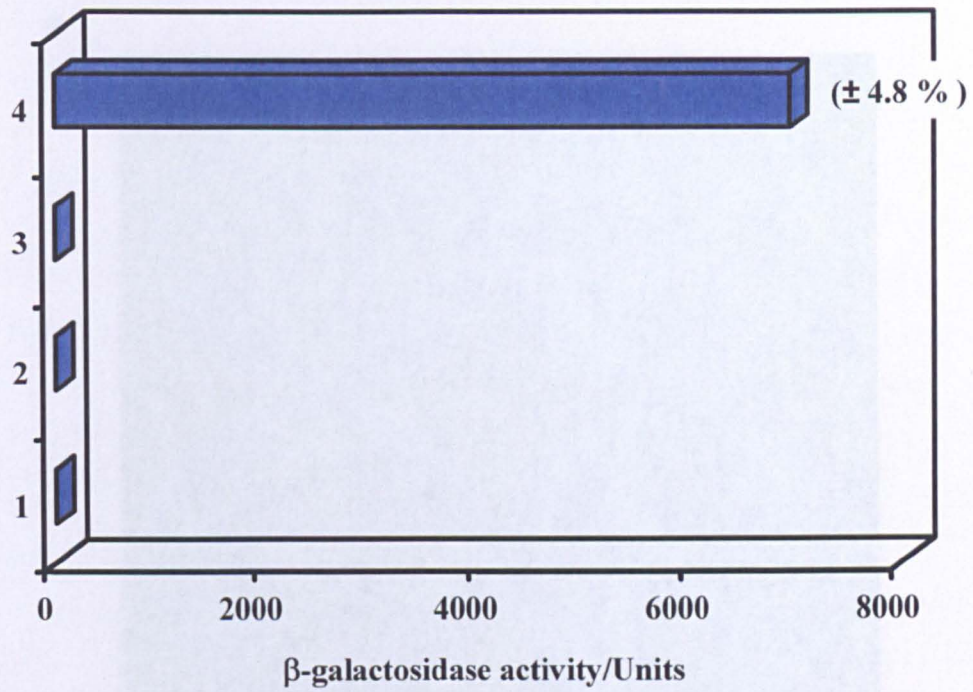
Cells were removed from high and low copper containing cultures during exponential growth phase and the β-galactosidase activity was measured quantitatively using the method described in Section 2.10.4 (Figure 4.18A). In addition, the integration of the promoter probe vector, pMHA034, into the 5' region of the *mmoX* σ^{54} promoter in *Mc. capsulatus* chromosome did not create a polar affect on the transcription of sMMO, since the expression of sMMO could be still detected using the naphthalene assay in a manner similar to the wild-type strain (Figure 4.18B).

The data presented in Figure 4.18 are similar to those presented in Figure 4.11, which showed the tight transcriptional regulation from the *mmoX* σ^{54} promoter. The β-galactosidase activity was massively upregulated under low copper-to-biomass ratio with only negligible activity under high copper-to-biomass ratio. In light of these data, the possibility of using *Mc. capsulatus* LacZ reporter strain as a high-throughput screen for the detection of sMMO mutants was investigated by cultivating the strain on NMS agar plates containing high and low concentrations of copper. The sensitivity of detection of expression of β-galactosidase activity was tested using MUG and X-gal as substrates (Figure 4.19).

The growth of the *Mc. capsulatus* LacZ reporter strain on X-gal dissolved in DMF was very poor with only slight improvement when grown on X-gal dissolved in

DMSO. Despite X-gal being an excellent substrate for detecting recombinant *E. coli* clones, the use of X-gal as a substrate for screening sMMO mutants in *Mc. capsulatus* was ruled out due to the relatively poor growth. However, the MUG reagent used as an alternative substrate yielded very promising results (Figure 4.19). Using the fluorogenic MUG reagent, the expression of β-galactosidase and thus sMMO expression could be detected with greater sensitivity and was reliably specific to sMMO-expressing cells. In addition, the background activity was minimal.

A.



B.

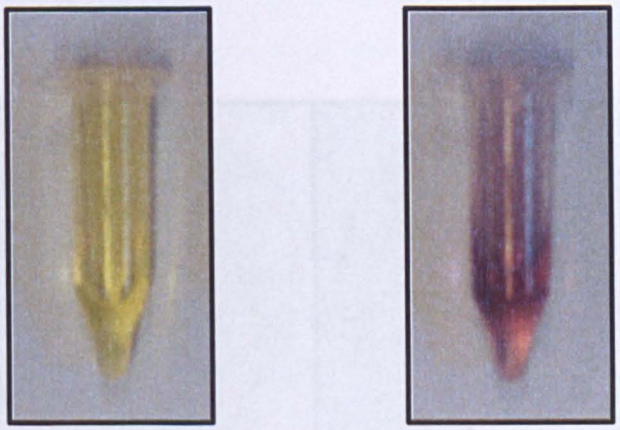
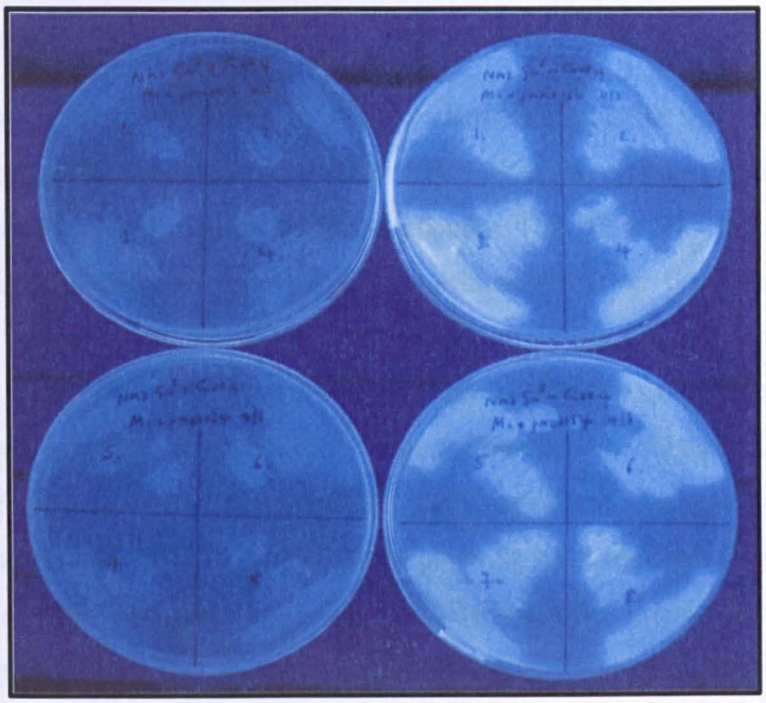


Figure 4.18 Qualitative and quantitative detection of β-galactosidase activity in *Mc. capsulatus*. A. Bar charts showing the activity of β-galactosidase in *Mc. capsulatus* wild-type strain (samples 1 and 2 used as controls) and LacZ reporter strain (samples 3 and 4) grown under high (samples 1 and 3) and low (samples 2 and 4) copper conditions. The activity represents a mean of four independent assays. The mean standard deviation (expressed as percentage) is shown in brackets. B. β-galactosidase activity using the ONPG assay on sample 4 following the conversion of o-nitrophenol-β-D-galactoside (ONPG) to o-nitrophenol (yellow product) (left tube). sMMO expression by sample four is shown by the naphthalene assay, which converts naphthalene to naphthol which in turn complexes with a zinc complex, and forming a purple diazo-compound (right tube).

A.



B.

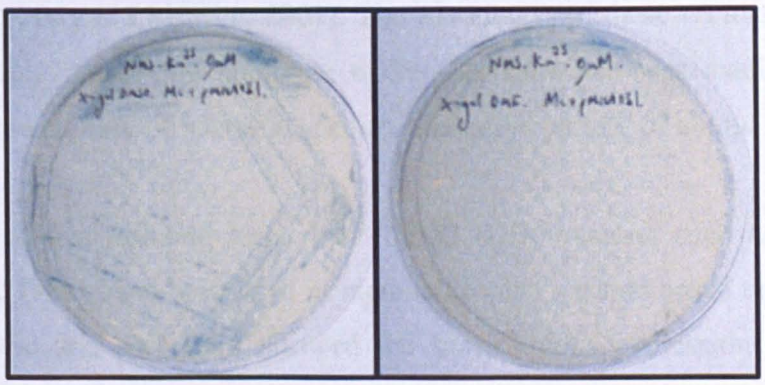


Figure 4.19 Detection of β-galactosidase activity in the *Mc. capsulatus* reporter strain using A. MUG assay performed on NMS agar plates containing copper (left hand side plates) and no added copper (right hand side plates). β-galactosidase activity is indicated by fluorescing white colonies. B. *Mc. capsulatus* LacZ reporter strain grown in the presence of X-gal (40 μg ml^{-1} each) dissolved in DMSO (left plate) and in DMF (right plate). The formation of blue colonies indicated β-galactosidase activity.

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4.5 Broad host range promoter probe vectors

4.5.1 Introduction

The use of broad host-host-range (BHR) vectors as genetic tools for cloning and expressing foreign genes in methanotrophs has lagged significantly behind those available for many other well studied bacterial species. The BHR RK2 vectors based on the IncP replicon is the most frequently used system, however their development for common use has not been so widespread since one of the major problems with these plasmids is their relative large size and the lack of DNA sequence information, thus limiting the ease of their manipulation. These problems were solved following the sequencing of the original 60-kb transmissible RK2 plasmid and the development of a number of derivative vectors based on minimal plasmid RK2 replicon (Blatny *et al.*, 1997a; Blatny *et al.*, 1997b; Santos *et al.*, 2001). These plasmids consisted of origin of vegetative replication (*oriV*) and the gene encoding the essential replication initiating protein, TrfA, which binds to *oriV* and thus initiates plasmid DNA replication. Similar BHR vectors utilising reporter genes were developed following the isolation of a spontaneous mutant of a small IncP plasmid for use in methylotrophs and other Gram-negative bacteria (Marx & Lidstrom, 2001). The advantages of these vectors were that they were relatively small, the complete nucleotide sequence was available, they replicated in a large number of bacterial species, and had a variety of unique restriction sites for cloning.

Previous studies utilising large IncP based BHR vectors, such as pVK100 (Knauf & Nester, 1982) have been used in methanotrophs. Vectors based on pVK100, such as pVK100Sc and pVK104 allowed the heterologous expression of sMMO operon in methanotrophs (Lloyd *et al.*, 1999a; Lloyd *et al.*, 1999b). However, the sMMO activity was much reduced in comparison to that of the wild-type strain.

4.5.2 Construction of the BHR promoter probe vectors, pMHA199 and pMHA200

To assess the usefulness of the BHR promoter probe vector pCM130 in methanotrophs, it required some modifications. The *tetR* and *tetA* gene in pCM130 conferring tetracycline resistance is a poor antibiotic selection marker in methanotrophs and therefore it was replaced by a kanamycin resistance gene, which was a suitable antibiotic marker. The *tetR* and *tetA* genes were removed from pCM130 following restriction digestion with *Sex*AI and *Nar*I and a DNA fragment carrying a kanamycin resistance gene was digested out of pCM132 with similar restriction enzymes and cloned into pCM130 to give the new BHR promoter probe vector, pMHA199 (Figure 4.20). In addition an alternative BHR promoter probe vector utilising *gfp* was constructed. The *lacZ* gene from pCM132 was removed by *Kpn*I and *Sph*I digestion and a *gfp* gene was cloned in via the same restriction sites to give the new vector, pMHA200 (Figure 4.20). Note: The *gfp* gene was amplified from pMJ153 (Figure 4.4) along with its natural RBS using primers *gfp-Kpn*I-F (5'-TCA TAT **GGT ACC** AAA ATA AGG AGG-3') and *gfp-Sph*I-R (5'-CGA ATA ATT CTA C**GC ATG C**TA TTT G-3'), which included restriction sites, as indicated by the primer names, to facilitate cloning.

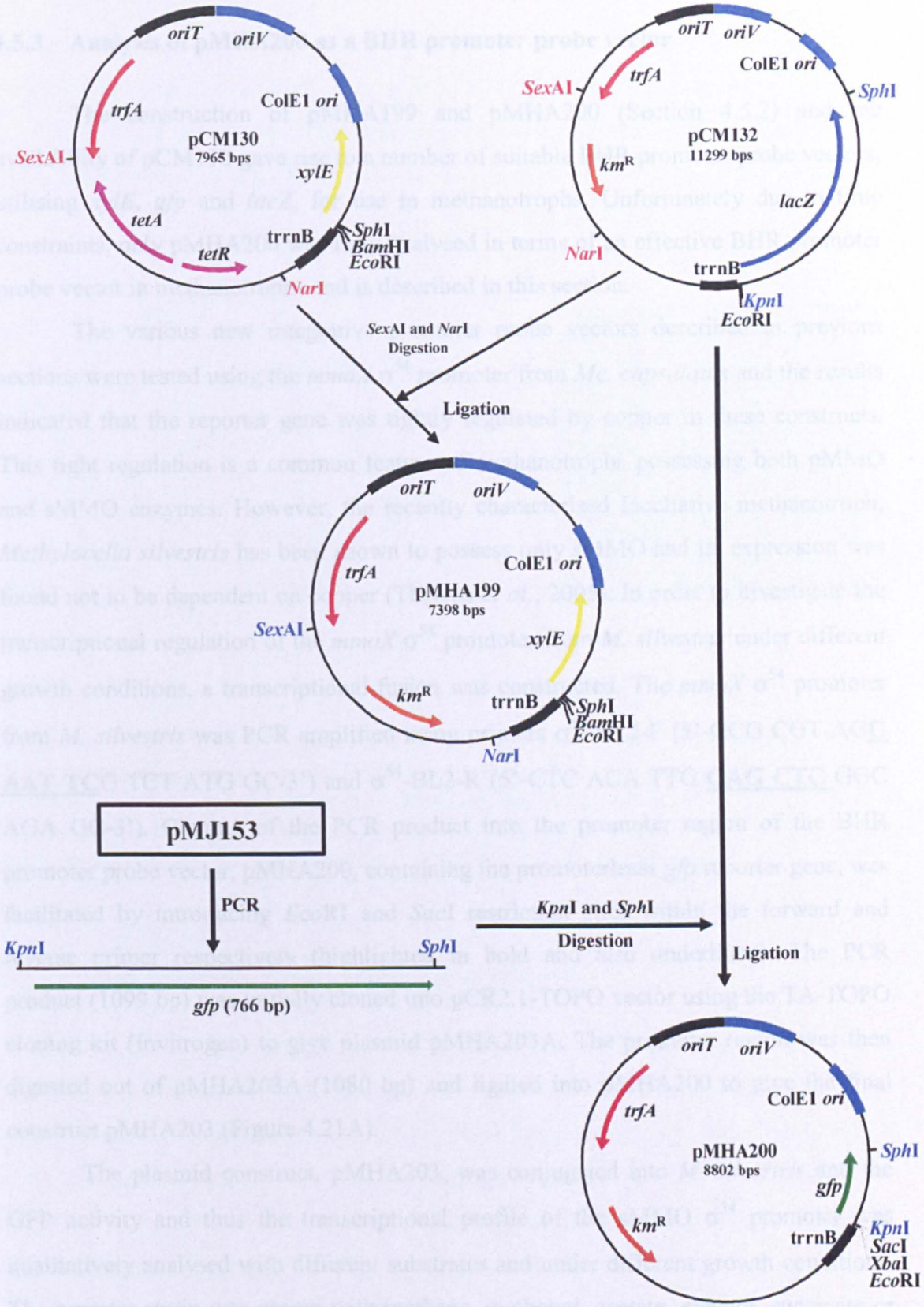

Figure 4.20 A schematic representation of the cloning steps involved in the construction of the BHR promoter probe vectors, pMHA199 and pMHA200. The restriction sites highlighted in red and blue are those involved in cloning.

4.5.3 Analysis of pMHA200 as a BHR promoter probe vector

The construction of pMHA199 and pMHA200 (Section 4.5.2) and the availability of pCM132 gave rise to a number of suitable BHR promoter probe vectors, utilising *xylE*, *gfp* and *lacZ*, for use in methanotrophs. Unfortunately due to time constraints, only pMHA200 was fully analysed in terms of an effective BHR promoter probe vector in methanotrophs and is described in this section.

The various new integrative promoter probe vectors described in previous sections were tested using the *mmoX* σ^{54} promoter from *Mc. capsulatus* and the results indicated that the reporter gene was tightly regulated by copper in these constructs. This tight regulation is a common feature of methanotrophs possessing both pMMO and sMMO enzymes. However, the recently characterised facultative methanotroph, *Methylocella silvestris* has been shown to possess only sMMO and its expression was found not to be dependent on copper (Theisen *et al.*, 2005). In order to investigate the transcriptional regulation of the *mmoX* σ^{54} promoter from *M. silvestris* under different growth conditions, a transcriptional fusion was constructed. The *mmoX* σ^{54} promoter from *M. silvestris* was PCR amplified using primers σ^{54}-BL2-F (5'-GCG CGT AG**G AAT TC**G TCT ATG GC-3') and σ^{54}-BL2-R (5'-CTC ACA TTG **GAG CTC** GGC AGA GC-3'). Cloning of the PCR product into the promoter region of the BHR promoter probe vector, pMHA200, containing the promoterlesss *gfp* reporter gene, was facilitated by introducing *Eco*RI and *Sac*I restriction sites within the forward and reverse primer respectively (highlighted in bold and also underlined). The PCR product (1099 bp) was initially cloned into pCR2.1-TOPO vector using the TA-TOPO cloning kit (Invitrogen) to give plasmid pMHA203A. The promoter region was then digested out of pMHA203A (1080 bp) and ligated into pMHA200 to give the final construct pMHA203 (Figure 4.21A).

The plasmid construct, pMHA203, was conjugated into *M. silvestris* and the GFP activity and thus the transcriptional profile of the sMMO σ^{54} promoter was qualitatively analysed with different substrates and under different growth conditions. The reporter strain was grown with methane, methanol, acetate, ethanol, succinate or pyruvate as the sole source of carbon and energy. In addition, the reporter strain was grown under high and low copper conditions with methane as the growth substrate. GFP fluorescence was only observed when the reporter strain was grown on methane under high and low copper conditions (Figure 4.21B), which clearly indicated that

transcription from the sMMO σ^{54} promoter is not repressed by copper ions in *M. silvestris*, but was switched off when grown on alternative carbon source (Theisen *et al.*, 2005). These data provided the first insights into the alternative mode of regulation of the expression of the sMMO enzyme in *M. silvestris*.

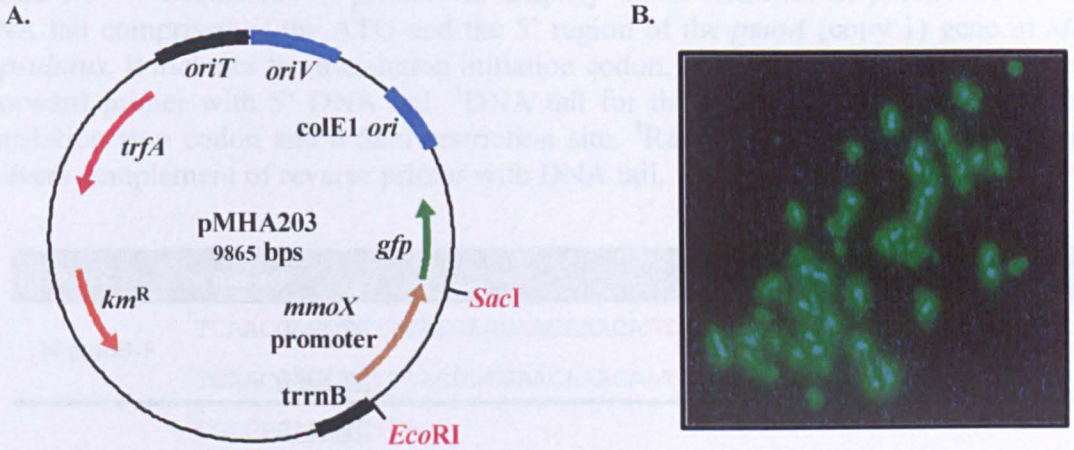

Figure 4.21 *M. silvestris* GFP reporter strain. A. Schematic representation of the BHR promoter probe vector pMHA203 containing the *mmoX* σ^{54} promoter from *M. silvestris* fused to a promoterless *gfp*. The *Eco*RI and *Sac*I restriction sites were used to facilitate the cloning of the promoter. B. GFP fluorescence of *M. silvestris* reporter strain grown on methane and visualised under a fluorescent microscope.

4.5.4 Over-expression of the N-terminus of pMmoB

To further demonstrate the usefulness of the BHR promoter probe vector, pMHA200, it was used as an over-expression vector. The N-terminus of MmoB of the pMMO enzyme (N-pMmoB) was hypothesised to play a role in forming a complex with methanol dehydrogenase (MDH) (Akimitsu Miyaji, personal communication). The MDH was thought to be involved in electron donation to the active site of pMMO via N-pMmoB, which is required for the initial activation of the C-H bond for methane oxidation. In order to test this hypothesis, the N-*pmmoB* was PCR amplified from *Mc. capsulatus* using primers N-pmoB-F and N-pmoB-R and fused to the pMMO σ^{70} promoter in plasmid pMHA201 (Chapter 6) via *Sac*I restriction sites to give the new construct, pMHA202 (Figure 4.22A). In order to selectively clone and express the N-

terminus of pMmoB in *E. coli*, DNA tails containing the translation initiation codon (ATG), RBS for pMMO σ^{70} promoter (GAGGA) and the translation stop codon (TAA) were included in the same coding frame as the pMmoB in the forward and reverse primer respectively (Table 4.4).

Table 4.4 Sequences of primers to amplify the N-terminus of *pmoB*. [1]A 31 bp DNA tail comprised of the ATG and the 5' region of the *pmoA* (copy 1) gene in *Mc. capsulatus*. It includes the translation initiation codon, RBS, and a *Sac*I restriction site. [2]Forward primer with 5' DNA tail. [3]DNA tail for the reverse primer containing the translation stop codon and a *Sac*I restriction site. [4]Reverse primer with 3' DNA tail. [5]Revers complement of reverse primer with DNA tail.

Primers	Sequence
N-pmoB-F	[1]TCAACGAGCTCTTTAGGAGGAACAAACAATG
	[2]TCAACGAGCTCTTTAGGAGGAACAAACAATGCACGGTGAGAAATCGCAGGC
N-pmoB-R	[3]GGCGAGAGCTCTTA
	[3]GGCGAGAGCTCTTATTCGTTGTAGTTCTCCAGGTCCAC
	[4]GTGGACCTGGAGAACTACAACGAATAAGAGCTCTCGCC

The plasmid construct pMHA202, containing the genes encoding N-pMmoB and GFP, which were under the transcriptional control of pMMO σ^{70} promoter, were over-expressed in *E. coli* S17.1 λ*pir*. The crude extracts, soluble and particulate fractions were isolated from exponentially growing *E. coli* cultures as described in Section 2.9.1 and the respective fraction were analysed on a 12.5 % (w/v) SDS-PAGE gel (Figure 4.22

B). Protein fractions were also isolated from *E. coli* S17.1 λ*pir* wild-type strain and strain containing plasmid pMHA201 for use as controls.

The data presented in Figure 4.22B clearly shows the over-expression of both GFP (27 kDa) and N-pMmoB (17 kDa) in *E. coli* using the BHR vectors, pMHA201 and pMHA202, which were both based on pMHA200 (Section 4.5.2). As expected the GFP, which is a soluble protein, was only present in the soluble fraction, whereas the N-pMmoB, which is a membrane protein, was present only within the particulate or insoluble fraction. In the case of the over-expression of GFP from pMHA201, it accounted for approximately 15 % of the total cellular protein, whereas the over-expression of both GFP and N-pMmoB from pMHA202 accounted for approximately 25 % of the total cellular protein.

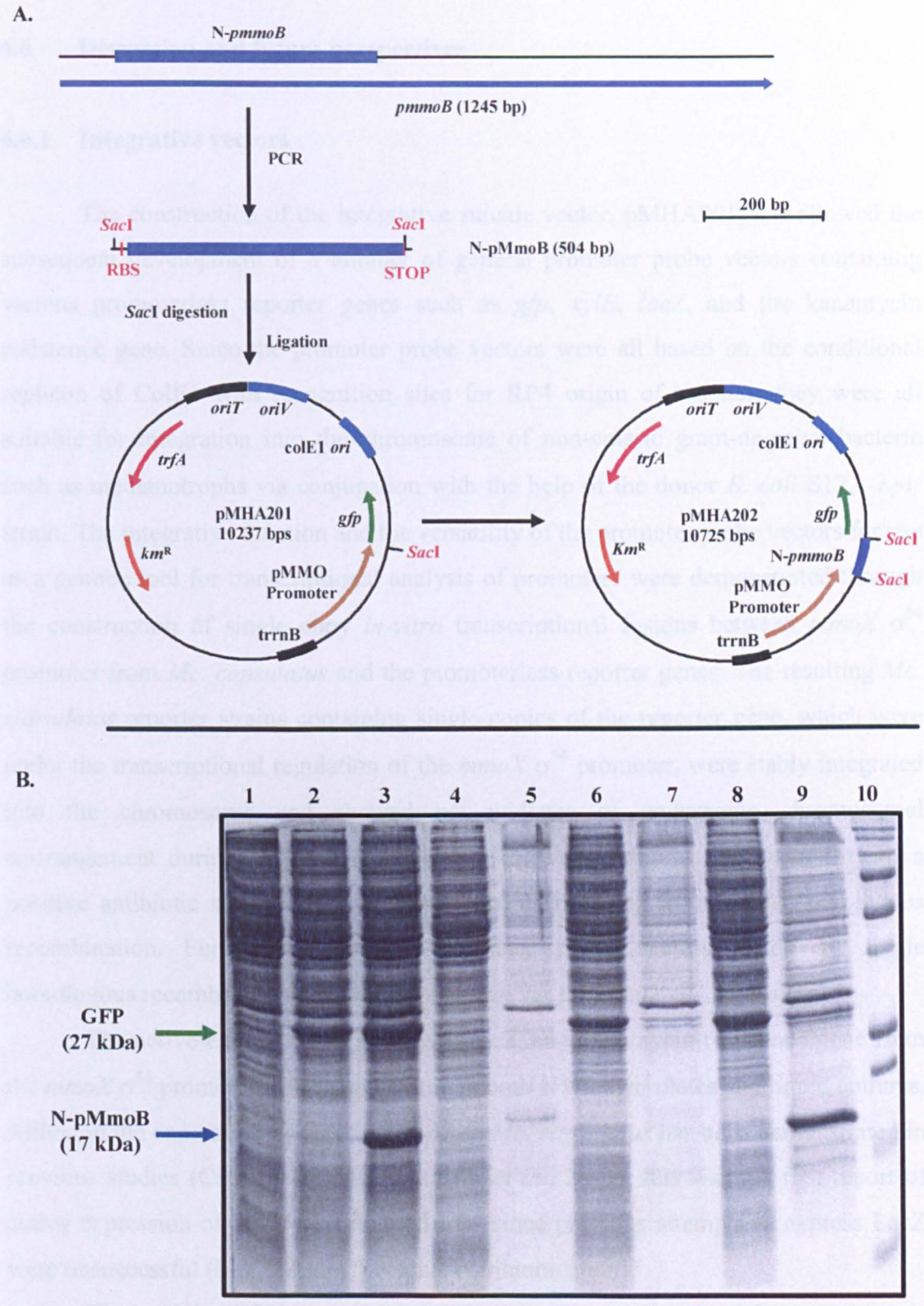

Figure 4.22 Over-expression of N-pMMOB in *E. coli*. A. Schematic representation of the strategy for constructing pMHA202. B. SDS-PAGE (12.5 % w/v) gel containing *E. coli* S17.1 λ*pir* crude extracts (lanes 1-3), soluble fractions (lanes 4, 6, and 8), and particulate fractions (lanes 5, 7, and 9) from wild-type strains (lanes 1, 4, and 5), strains containing plasmid pMHA201 (lanes 2, 6, and 7) and strains containing plasmid pMHA202 (lanes 3, 8, and 9). Lane 10 contains a molecular mass marker (Dalton Mark VII-L – Sigma).

4.6 Discussion and future perspectives

4.6.1 Integrative vectors

The construction of the integrative suicide vector, pMHA001, has allowed the subsequent development of a number of general promoter probe vectors containing various promoterless reporter genes such as *gfp*, *xylE*, *lacZ*, and the kanamycin resistance gene. Since the promoter probe vectors were all based on the conditional replicon of ColE1 with recognition sites for RP4 origin of transfer, they were all suitable for integration into the chromosome of non-enteric gram-negative bacteria such as methanotrophs via conjugation with the help of the donor *E. coli* S17.1 λ*pir* strain. The integrative function and the versatility of the promoter probe vectors for use as a genetic tool for transcriptional analysis of promoters were demonstrated through the construction of single copy *in-vitro* transcriptional fusions between *mmoX* σ^{54} promoter from *Mc. capsulatus* and the promoterless reporter genes. The resulting *Mc. capsulatus* reporter strains containing single copies of the reporter gene, which were under the transcriptional regulation of the *mmoX* σ^{54} promoter, were stably integrated into the chromosome and showed no evidence of undergoing chromosomal rearrangement during periods of continuous cultivation due to the availability of a positive antibiotic selection for the recombinants resulting from single homologous recombination. Furthermore, all the promoter probe vectors underwent single homologous recombination with high frequency via the *mmoX* σ^{54} promoter.

The active expression of *gfp*, *xylE*, *lacZ*, and kanamycin resistance gene from the *mmoX* σ^{54} promoter was demonstrated in both NMS agar plates and liquid cultures. Although the expression of GFP and XylE in *Mc. capsulatus* has been demonstrated in previous studies (Csaki *et al.*, 2003; Stolyar *et al.*, 2001), this was the first report of active expression of LacZ in methanotrophs since previous attempts to express LacZ were unsuccessful (M. Lidstrom, personal communication).

The activity of the reporter gene, with the exception of kanamycin (see discussion below) resembled the activity of sMMO enzyme under high and low copper conditions and the data obtained were comparable to those obtained in previous studies (Csaki *et al.*, 2003; Nielsen *et al.*, 1996). During growth under high copper-to-biomass ratio, the transcription from the *mmoX* σ^{54} promoter was totally repressed and the reporter gene activity detected was negligible, which also indicated that there was no

interference from background transcription from the plasmid or indeed from promoters located 5' of the integrated promoter probe vector. In addition, the introduction of a second copy of the promoter into the chromosome did not create a polar affect on the transcription from the original promoter and therefore did not affect sMMO expression. This demonstrated the usefulness of these promoter probe vectors for analysing promoter activities under different growth conditions and thus fulfilled partially the initial aims of this study.

In addition to the initial aims of this study, the usefulness of the various reporter genes as a selection marker for the high-throughput detection of sMMO expression were assessed. Overall the activity of the reporter genes in *Mc. capsulatus* was relatively low compared to the activity in *E. coli* due to the single copy integration. In particular the GFP activity was low, even when cultivated to high cell density in a 5 L fermentor compared to the XylE and LacZ activity in *Mc. capsulatus*. The relatively low level of GFP expression made it unsuitable as a reporter strain for detecting sMMO expression. One of the contributing factors for this low level of GFP expression was possibly due to the RBS of GFP (AGGAG), which differed from that of *mmoX* (GAGGA) and was possibly only poorly recognised in *Mc. capsulatus*. Subtle differences in the RBS have been demonstrated experimentally to be sufficient to reduce the binding affinity of the ribosomal RNA for the RBS and thus the efficiency of translation (Schottel *et al.*, 1984). In summary it can be concluded that the low GFP expression is due to an effect post transcription since exactly the same promoter region was used in the XylE and LacZ construct, where high levels of reporter gene expression were observed. The only difference was that in these constructs, the RBS were altered to resemble that of *mmoX*. For the use in future studies, it may be possible to improve the GFP activity by constructing exact transcriptional fusions with the promoter by incorporating a DNA tail, which includes the DNA region 5' of the ATG of the gene of interest including the native RBS, similarly to the construction of the kanamycin and LacZ transcriptional fusions carried out in this study (Sections 4.3 and 4.4).

In comparison, the XylE reporter strain yielded very high levels of reporter gene activity when cultivated to high cell densities in a 5 L fermentor under low copper conditions, which were almost comparable to the activities yielded from *E. coli* cultures cultivated in flasks (Figure 4.11 and 4.12). On NMS agar plates the XylE activity was detectable using the chromogenic substrate, catechol. In addition, the cells

expressing XylE could be easily differentiated from those that were not and thus made it a useful reporter strain for detecting sMMO expression.

Similarly, the LacZ reporter strain yielded very high levels of reporter gene activity (Figure 4.18). The use of X-gal as a chromogenic substrate for the detection of sMMO expression was unsuccessful due to the poor growth on X-gal dissolved in DMF or DMSO and therefore it was not possible to exploit the blue/white screen for detecting sMMO expression. This was overcome by using the fluorogenic substrate, MUG, which when hydrolysed emitted a blue fluorescence under UV light (Figure 4.19). Although the activity of both XylE and LacZ using catechol and MUG as substrate, respectively, were accumulative, the blue fluorescence emitted following the hydrolysis of MUG was detectable with greater sensitively compared to the yellow diffusible product produced following the hydrolysis of catechol. This made the *Mc. capsulatus* LacZ strain an excellent reporter strain for detecting sMMO expression and thus for the high-throughput detection of sMMO mutants.

The rationale for constructing a antobiotic-resistance promoter probe vector, such as kanamycin, was to allow the direct selection of sMMO mutants (i.e. sMMO constitutive mutants where the transcription of the *mmoX* σ^{54} promoter will not be repressed under high copper conditions). Following the initial attempts to isolate *Mc. capsulatus* kanamycin reporter strains on kanamycin (15 $\mu g\ ml^{-1}$) and gentamicin (5 $\mu g\ ml^{-1}$) were unsuccessful since the initial selection on kanamycin made it lethal due to the repression of the promoter and thus the expression of the protein conferring kanamycin resistance. In conclusion, the unsuccessful nature of this experiment was most likely due to the tight regulation of the *mmoX* σ^{54} promoter with respect to copper ions and in turn reinforced the data already obtained using the GFP, XylE and LacZ reporter strain. Isolation of the kanamycin reporter strain will be very useful for future studies for the direct detection of sMMO mutants and can be relatively easily isolated by initially selecting for single cross-over recombination events via the *mmoX* promoter on gentamicin and then subsequently on both kanamycin and gentamicin following the induction of the promoter under low copper conditions.

4.6.2 Broad-host range vectors

The construction of the low background BHR promoter probe vectors pCM130 and pMC132 by Marx and Lidstrom, (2004) made it possible to construct a modified version of these vectors suitable for use in methanotrophs. The BHR promoter probe vectors pMHA199 and pMHA200 containing *xylE* and *gfp* respectively were constructed. These vectors were based on the BHR RK2 vectors containing the IncP replicon. The BHR replication features and the adaptability into species specific promoter probe vector was demonstrated through the construction of a *in-vitro* transcriptional fusion using plasmid pMHA200 and *mmoX* σ^{54} promoter from *M. silvestris*. The resulting vector, pMHA203, was successfully transferred and maintained in *M. silvestris* and the GFP activity was used to assay the transcriptional activity of the promoter under different growth conditions and copper concentrations. The data obtained using this vector yielded valuable information about the transcriptional regulation of the *mmoX* σ^{54} promoter from *M. silvestris*, which have been published recently (Theisen *et al.*, 2005). Most importantly, it confirmed at the level of transcription the constitutive nature of the *M. silvestris mmoX* σ^{54} promoter under high and low copper conditions. It is noteworthy that vectors based on RK2 are relatively low in copy number, with an estimated copy number of 5-7 per chromosome in *E. coli*. However, the GFP activity of the multi-copy plasmid, pMHA203, in *M. silvestris* was found to be significantly higher than that of the single integrated copy of GFP in *Mc. capsulatus* using vector pMHA011. Recent work by Andreas Theisen is focused on the quantification of the GFP activity in *M. silvestris*. In addition the GFP reporter strain is being further exploited to assay sMMO expression following the construction of knock-out mutants in *mmoR*, since in *M. silvestris* the regulatory genes *mmoR* and *mmoG* are co-transcribed with the sMMO structural genes (Theisen *et al.*, 2005).

The feasibility of using these vectors in over-expression studies were also demonstrated in *E. coli* following the selective over-expression of the N-terminus of pMmoB for the use in *in-vitro* protein binding studies with MDH (Figure 4.22). Recent work by Akimitsu Miyaji is focused on the purification and the re-folding of the N-terminus of pMmoB. In light of the success using one of these vectors for expressing in *E. coli*, it may be possible to use them for over-expressing proteins of interest in methanotrophs.

4.6.3 Summary and future perspectives

In summary, in this study a series of integrative suicide promoter probe vectors and BHR promoter probe vectors have been constructed. Through the construction of transcriptional fusions with *mmoX* σ^{54} promoter from *Mc. capsulatus* and *M. silvestris* the usefulness of these vectors have been demonstrated. All the promoter probe vectors constructed in this study are relatively small in size containing a large selection of unique cloning sites and with the availability of the full sequence information which allowed them to be readily adaptable to species specific promoter probe or expression vectors. The availability of these vectors has greatly facilitated further detailed studies to be carried out to investigate the molecular regulation of MMO in methanotrophs. For example the LacZ reporter strain has been shown to be a very useful reporter gene since its activity was detectable with very high sensitively using the MUG substrate and therefore it was further exploited for the high-throughput detection of sMMO mutants following transposon mutagenesis in Chapter 6 and in further MMO regulation studies described in Chapter 5.

The availability of the genome sequence of *Mc. capsulatus* has led to the identification of a number of targets for mutagenesis. The current procedure for constructing mutations is based on the marker-exchange mutagenesis developed originally by Martin and Murrell (Martin & Murrell, 1995). However, as it can be seen from the work presented in Chapter 3, this method for constructing mutations involved several cloning steps and was very time consuming. The construction of the integrative suicide vector, pMHA001, in this study could be theoretically used to construct mutants by insertional inactivation. Since the vector is conditional for its replication, it can be used to create a defined duplication within a target genome as was shown with the *mmoX* σ^{54} promoter in the above reporter strains. For example this system can also be used to obtain gene disruptions by cloning an internal portion of a gene sequence into such a suicide plasmid and thus generating two incomplete gene copies upon integration of the plasmid into the chromosome (Figure 4.23). Construction of the vector for obtaining a mutant in a defined gene using this system will only involve one cloning step and thus will allow many more gene disruptions to be carried out. It is noteworthy that although gene disruption using this system has not been demonstrated in this study, all the steps necessary for constructing mutants have been already demonstrated through the integration of the various reporter genes in *Mc. capsulatus*,

which was used to create a duplicate copy of the *mmoX* σ^{54} promoter (Figure 4.8). Similar suicide vectors have been used for insertional inactivation of genes in *Rhizobium meliloti* (Becker *et al.*, 1995) and *Rhodobacter sphaeroides* (Penfold & Pemberton, 1992).

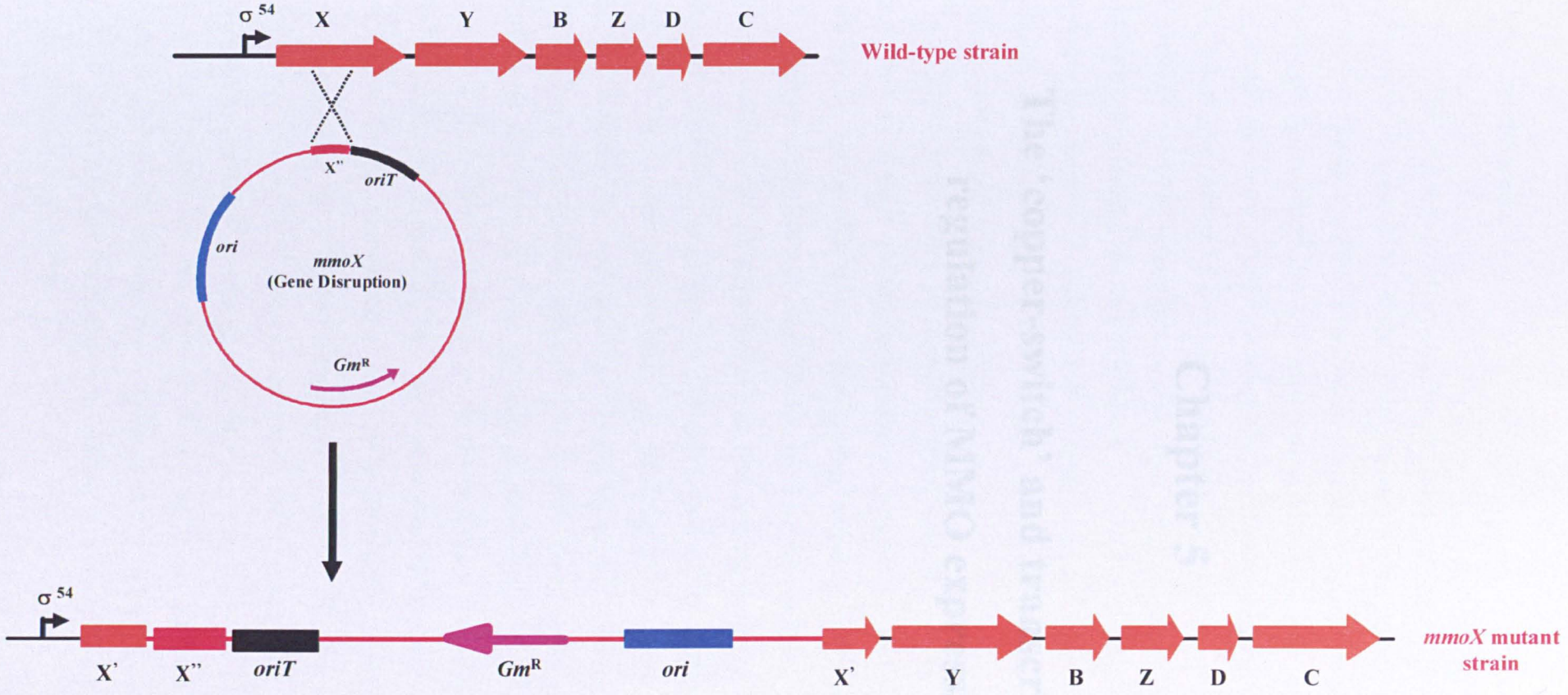

Figure 4.23 An example gene mutation (i.e. *mmoX*) by insertional inactivation system using an integrative suicide vector. A hypothetical arrangement of the structural genes encoding sMMO (*mmoXYBZDC*) following single homologous recombination between internal portions of the *mmoX* gene from the plasmid and the chromosome and thus producing two incomplete copies of the *mmoX* gene. X' represents partial chromosomal copy of *mmoX* and X'' represents the internal portion of *mmoX* gene on the plasmid. The black line represents the DNA sequence of the chromosome and the red line represents the DNA sequence of the plasmid. Note: The orientation of the cloned internal portion of the gene will not affect recombination.

Chapter 5

The 'copper-switch' and transcriptional regulation of MMO expression

5.1 Introduction

The regulation of expression of bacterial genes responsible for cellular adaptation to a particular niche, their response to environmental stress, stimuli and growth is highly complex. In addition, the complexity of the molecular mechanisms involving gene regulation is further increased since they are often subjected to regulation at various levels involving additional factors in the form of activators and repressors and thus are often not well understood. However, a great deal of information is known about the transcriptional regulation of gene expression in *Escherichia coli* (Burgess & Anthony, 2001; Kazmierczak *et al.*, 2005; Murakami & Darst, 2003). In general, transcription mediated by DNA-dependent RNA polymerase is regarded as the critical first step in regulation, where DNA is transcribed into RNA and is the target for many regulators of gene expression.

In bacteria, RNA polymerase is responsible for most cellular RNA synthesis and the core RNA polymerase complex ($\alpha_2\beta\beta'$) is sufficient for transcription elongation and termination but not initiating transcription (Paget & Helmann, 2003). The efficient initiation of transcription requires an additional subunit called the σ factor, which forms the RNA polymerase holoenzyme ($E\sigma$). The $E\sigma$ plays the essential role of promoter recognition and the formation of an competently active enzyme complex, thus allowing initiation of transcription (Burgess *et al.*, 1969). The specificity of $E\sigma$ for a particular promoter is governed by dissociable σ factors, which in bacteria are categorised into two main classes, based on their sequence similarities and mechanism of transcription initiation (Kazmierczak *et al.*, 2005).

The σ^{70}-class of σ factors are the major class of σ factors and are often referred to as primary σ factors as they are responsible for the expression of most genes expressed during exponential growth (Wigneshweraraj *et al.*, 2001). Activation of transcription by holoenzyme containing σ^{70} ($E\sigma^{70}$) is directed to a promoter element centred at -10 and -35 relative to the +1 transcription start site. Once bound, it undergoes conformation changes to form an open complex leading to transcription initiation without the requirement of an additional enhancer element (Paget & Helmann, 2003). In methanotrophs, the promoter initiating transcription of the pMMO operon (*pmoCAB*) has been shown to initiate from a σ^{70} like promoter located 5' of *pmoC* (Gilbert *et al.*, 2000).

The second class of σ factor, σ^{54}, is structurally and functionally distinct from those related to the primary σ^{70} family and is involved in the transcription of many different and unrelated genes (Cases *et al.*, 2003). In general, gene regulation by σ^{54} promoters is not essential for survival and growth under favourable conditions (Buck *et al.*, 2000). The mechanism of transcription from $E\sigma^{54}$ is different to that of $E\sigma^{70}$ in that it requires an enhancer binding protein (also known as the σ^{54}-dependent transcriptional activator) to promote isomerisation of the $E\sigma^{54}$-DNA complex to a transcription-competent open complex (Buck *et al.*, 2000). As shown in Chapter 3, analysis of the promoter region located 5' of *mmoX* in various methanotrophs (Table 3.2) revealed the presence of the highly conserved -24 and -12 consensus sequence for the σ^{54} promoter. In addition, the identification of a number of σ^{54}-dependent transcriptional activators (*mmoR*) specific for the sMMO σ^{54} promoter lent further support for a *bona fide* σ^{54} promoter driving transcription of the sMMO operon.

The phenomenon of the 'copper-switch' in methanotrophs, as discussed in Chapter 1 in some detail, was originally proposed by Stanley *et al.*, (1983) where the intracytoplasmic location of MMO activity was shown to be dependent on the copper to biomass ratio (Stanley *et al.*, 1983). Since this key finding, a number of studies were initiated in our laboratory to resolve the molecular mechanisms underlying the regulation of the expression of MMO by copper ions. Despite significant advances in biotechnology and our understanding of the 'copper-switch', the exact molecular mechanism regulating the expression of MMO is unknown. It is now generally accepted that the predominant intracytoplasmic location of MMO activity is dependent on the copper-to-biomass ratio. However, there is much debate surrounding the transcriptional regulation of the pMMO operon (Choi *et al.*, 2003; Nielsen *et al.*, 1997; Stolyar *et al.*, 2001) and the mechanism regulating the transcriptional on-off switch of the sMMO operon via the σ^{54} promoter is unknown. Furthermore, very little is known about the mechanisms involving the transport of copper in and out of the cell.

The initial aim of this study was to utilise the genetic tools developed in Chapter 4 to explore the 'copper-switch', particularly the transcriptional regulation of the pMMO σ^{70} promoter and the sMMO regulatory genes, *mmoR* and *mmoG*. Secondly, attempts were made to identify upstream activating sequence of the sMMO σ^{54} promoter by error-prone PCR. In addition, a 'Knock-in' mutagenesis approach was taken to overcome the tight regulation of transcription of the sMMO operon by

attempting to replace the sMMO σ^{54} promoter for a strong constitutive promoter. The sequencing of the *Mc. capsulatus* genome led to the direct identification of a number of homologues of copper transport proteins. Therefore, in order to exploit this information and to correlate their function to copper transport and thus in MMO regulation, knock-out mutants were constructed by marker-exchange mutagenesis and attempts were made to phenotypically characterise the resulting mutants.

5.2 Exploring the 'copper-switch'

5.2.1 Transcriptional activity of the pMMO σ^{70} promoter

The initial findings from the MMO regulation studies carried out on *Ms. sporium* (Chapter 3) indicated that the pMMO operon (*pmoCAB*) is transcribed constitutively and that it was regulated at a level post-transcription. These results are contrary to those reported by Choi *et al.*, (2003) and Stolyar *et al.*, (2001). Therefore, it was necessary to reinvestigate using various methods whether transcription of the *Mc. capsulatus* pMMO operon is regulated by copper and if it is linked to the 'copper-switch' transcription of the sMMO operon. To address these questions, firstly a transcriptional fusion was constructed with *gfp* in the BHR promoter probe vector pMHA200, and pMMO (operon 1) σ^{70} promoter from *Mc. capsulatus*. The pMMO σ^{70} promoter was PCR amplified using primers pMMO-σ^{70}-promoter-*Eco*RI-F (5'CGT TGA **GGAATT C**GG CAC AGA AAG TG-3') and pMMO-σ^{70}-promoter-*Sac*I-R (5'-TTT TTG TTC CTC CTA AA**GAGCTC** G TTG AC-3'), which included restriction sites, as indicated by the primer names, to facilitate cloning. It was difficult to amplify the promoter using these primers and only very faint products were obtained. The faint PCR products of the pMMO σ^{70} promoter were cloned into pCR2.1-TOPO vector, yielding the intermediate construct, pMHA201A. The promoter fragment was released with *Eco*RI and *Sac*I and cloned into pMHA200 via the same restriction sites, yielding the new vector, pMHA201 (Figure 5.1A). Electrocompetent *E coli* S17.1 λpir cells were transformed with pMHA201 and GFP expression was detected (Figure 5.1B) indicating that the pMMO σ^{70} promoter was recognised by *E. coli* RNA polymerase.

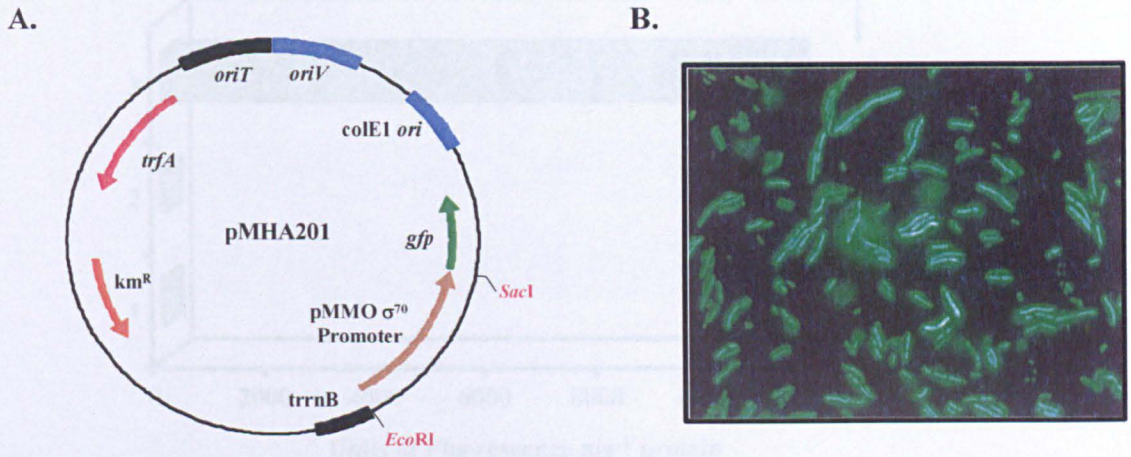

Figure 5.1 A. Schematic representation of the BHR promoter probe vector, pMHA201, containing the pMMO σ^{70} promoter from *Mc. capsulatus* fused to the promoterless *gfp*. B. GFP fluorescence of *E. coli* S17.1 λ*pir* cells, containing pMHA201, visualised under a fluorescent microscope.

The BHR promoter probe vector pMHA200 was based on pCM132, which contains a transcriptional terminator located 5' of the promoter region and was demonstrated to have low background reporter gene activity in *Methylobacterium extorquens* AM1 (Marx & Lidstrom, 2001). Since the pMMO σ^{70} promoter was recognised by *E. coli* RNA polymerase, it gave a convenient means to further investigate the background activity of the reporter gene in *E. coli* before embarking on detailed transcriptional regulation studies in *Mc. capsulatus*. This was accomplished by quantifying the GFP activity in cell-free extracts obtained from exponentially growing *E. coli* S17.1 λ*pir* cells containing pMHA200 (contains no promoter) and pMHA201 (contains pMMO σ^{70} promoter), respectively (Figure 5.2).

The data obtained confirmed the low background reporter gene activity of vectors based on pCM132 as no GFP activity was detected in *E. coli* cells containing pMHA200, whereas in *E. coli* cells containing pMHA201, high levels of GFP activity were detected. The low background property of these vectors made it ideal for their use in assaying promoter activity under different copper conditions.

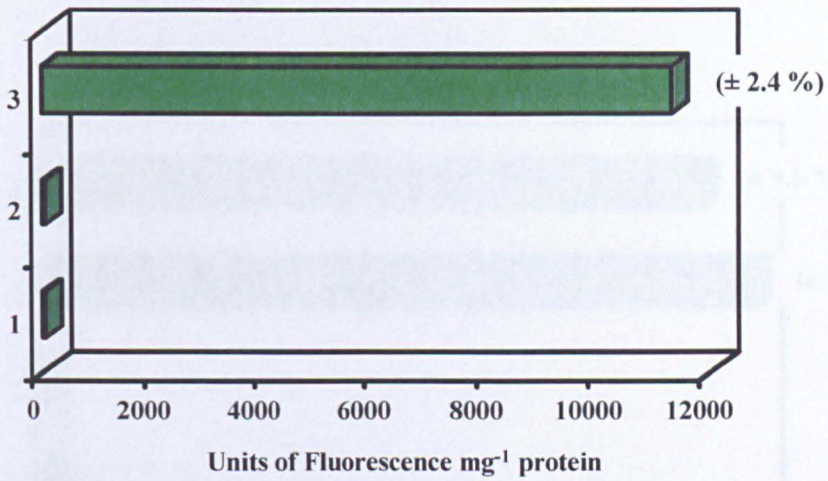

Figure 5.2 Bar chart showing the low background GFP activity of the BHR promoter probe vector, pMHA200 in *E. coli* S17.1 λ*pir*. The GFP activity was measured in cell-free extracts obtained from *E. coli* cells containing pMHA200 (sample 2) and pMHA201 (sample 3). Cell-free extracts extracted from wild-type strain was used as a control. The GFP activity represents a mean of two independent assays and the mean standard deviation (expressed as percentage) is shown in brackets.

To assay the transcriptional activity of the pMMO σ^{70} promoter in *Mc. capsulatus* under high (1 μM $CuSO_4$) and low (no added $CuSO_4$) concentrations of copper, pMHA201 was conjugated into *Mc. capsulatus*. As an additional control, pMHA200 was also conjugated into *Mc. capsulatus*. The resulting transconjugants were selected on NMS agar plates containing kanamycin (15 μg ml^{-1}). Following the initial qualitative analysis, GFP activity was detected in *Mc. capsulatus* containing pMHA201 grown under high and low concentrations of copper and as expected, no GFP activity was detectable in *Mc. capsulatus* containing pMHA200. In order to quantify the GFP activity driven from the pMMO σ^{70} promoter, both *Mc. capsulatus* reporter strains were cultivated in 5 L fermentors in batch cultures under high and low copper conditions. The naphthalene assay for sMMO was performed on cells from which cell-free extracts were prepared. The assay confirmed the presence of sMMO activity under low copper conditions and the absence of activity under high copper conditions. The specific GFP activities of the above samples were quantified using methods described in Section 2.10.2 (Figure 5.3).

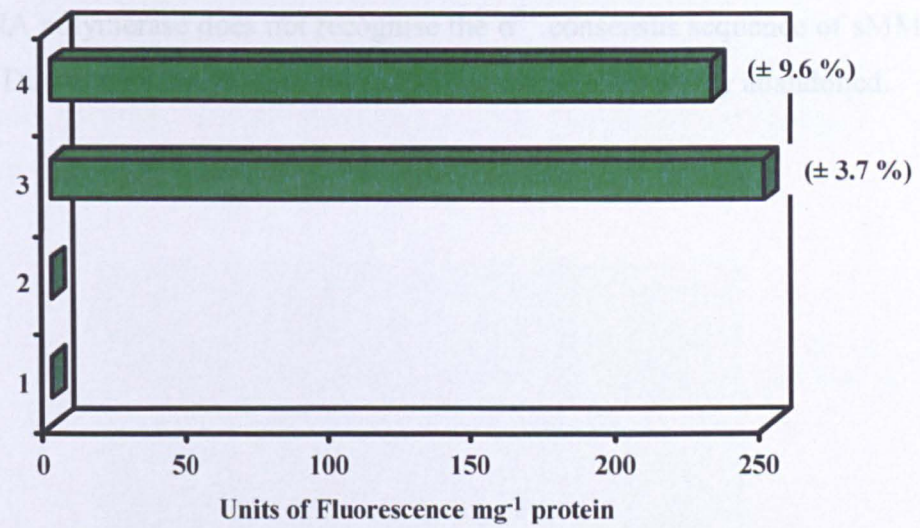

Figure 5.3 Bar chart showing the specific activity of GFP driven from pMMO σ^{70} promoter under high and low copper conditions. The GFP activity was measured from cell-free extracts extracted from *Mc. capsulatus* cells containing pMHA200 (sample 1 and 2) and pMHA201 (sample 3 and 4) grown under high (samples 2 and 4) and low (samples 1 and 3) copper conditions.

The data presented in Figure 5.3 indicated that transcription initiated from pMMO σ^{70} promoter is not affected by copper concentration. These data agreed with those obtained from *Ms. sporium* using RNA dot-blotting presented in Chapter 3.

In a separate experiment, a similar vector to pMHA201 was constructed by cloning the sMMO σ^{54} promoter from *Mc. capsulatus* into pMHA200 via *Eco*RI and *Sac*I sites to yield the vector, pMHA204. The promoter was amplified using primers *Mc*-σ^{54}-*Eco*RI-F (5'-GTG ATG GAA TTC GCG AGC ATA TCC-3') and *Mc*-σ^{54}-*Sac*I-R (5'-GAA TGA TGA GAG CTC GAT GAC GTC-3'). Since the transcriptional terminator in pMHA200 was very effective in blocking background transcription initiated from promoters present on the vector (Figure 5.2), it was convenient for investigating whether the tight transcriptional regulation of the sMMO σ^{54} promoter was a result of direct inhibition of transcription by copper. Therefore the purpose of constructing pMHA204 was to investigate the copper effect on the sMMO σ^{54} promoter in *E. coli*. However, it is noteworthy that once pMHA204 was introduced into *E. coli*, no GFP expression was observed. This is not unexpected as the σ^{54}

dependent transcriptional activator (MmoR) is not present in *E. coli* and therefore no transcription would be initiated from this promoter. In addition, it is possible that the *E. coli* RNA polymerase does not recognise the σ^{54} consensus sequence of sMMO σ^{54} promoter. Due to time constraints, this experiment was temporarily abandoned.

5.2.2 Transcriptional analysis of MMO and MMO regulatory genes

To further investigate the MMO 'copper-switch' at the level of transcription, RT-PCR and RNA dot-blot analysis were performed. Total RNA was extracted from exponentially growing fermentor cultures of *Mc. capsulatus* expressing pMMO or sMMO, using the method described in Section 2.3.2 (Figure 5.4).

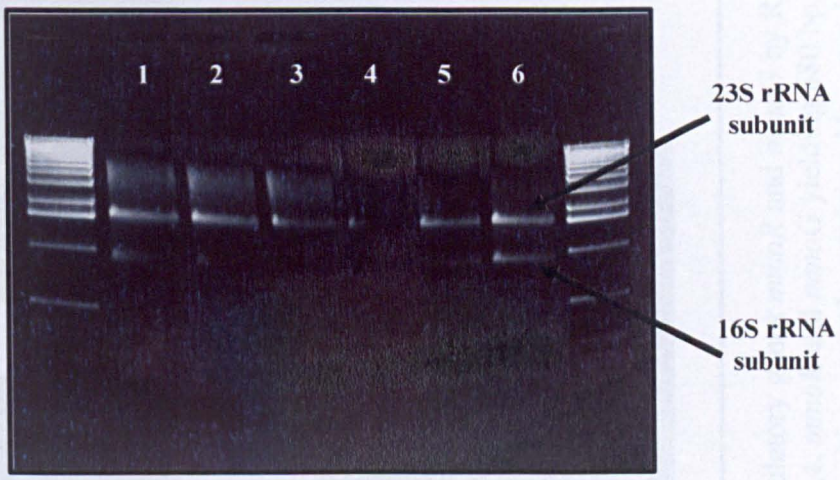

Figure 5.4 RNA extracted from *Mc. capsulatus* cultures expressing pMMO (samples 1-3) and sMMO (samples 4-6).

DNA from the RNA samples was removed by treatment with DNaseI and the removal of DNA was confirmed by PCR (Figure 5.5A). RT-PCR, as described in Section 2.6.3, was performed to monitor mRNA transcripts for *mmoX*, *pmoA*, *mmoR*, and *mmoG* under high and low copper conditions (Figure 5.5B-E). It is noteworthy that since the transcription profile of *mmoX* was shown (Chapter 3 and 4) to be tightly regulated by copper at the transcriptional level, *mmoX* RT-PCR in this experiment acted as an internal standard to confirm low copper or sMMO expressing conditions of the RNA extracted. The *mmoX* and *pmoA* RT-PCR was performed using primers described previously (Holmes *et al.*, 1995; Hutchens *et al.*, 2004) and *mmoR* and *mmoG* RT-PCR was done using primers *mmoR*-F (5'-GAA GTG GCC GAA GCC TGG TG-3'), *mmoR*-R (5'-CAG CTC GCT CTC GAT GAG GTC-3'), *mmoG*-F (5'-GGT GGC TAA CTC CAT CGT ACT C-3') and *mmoG*-R (5'-ACC GCG ACG GCC TTG AAG AC-3') which were designed in this study.

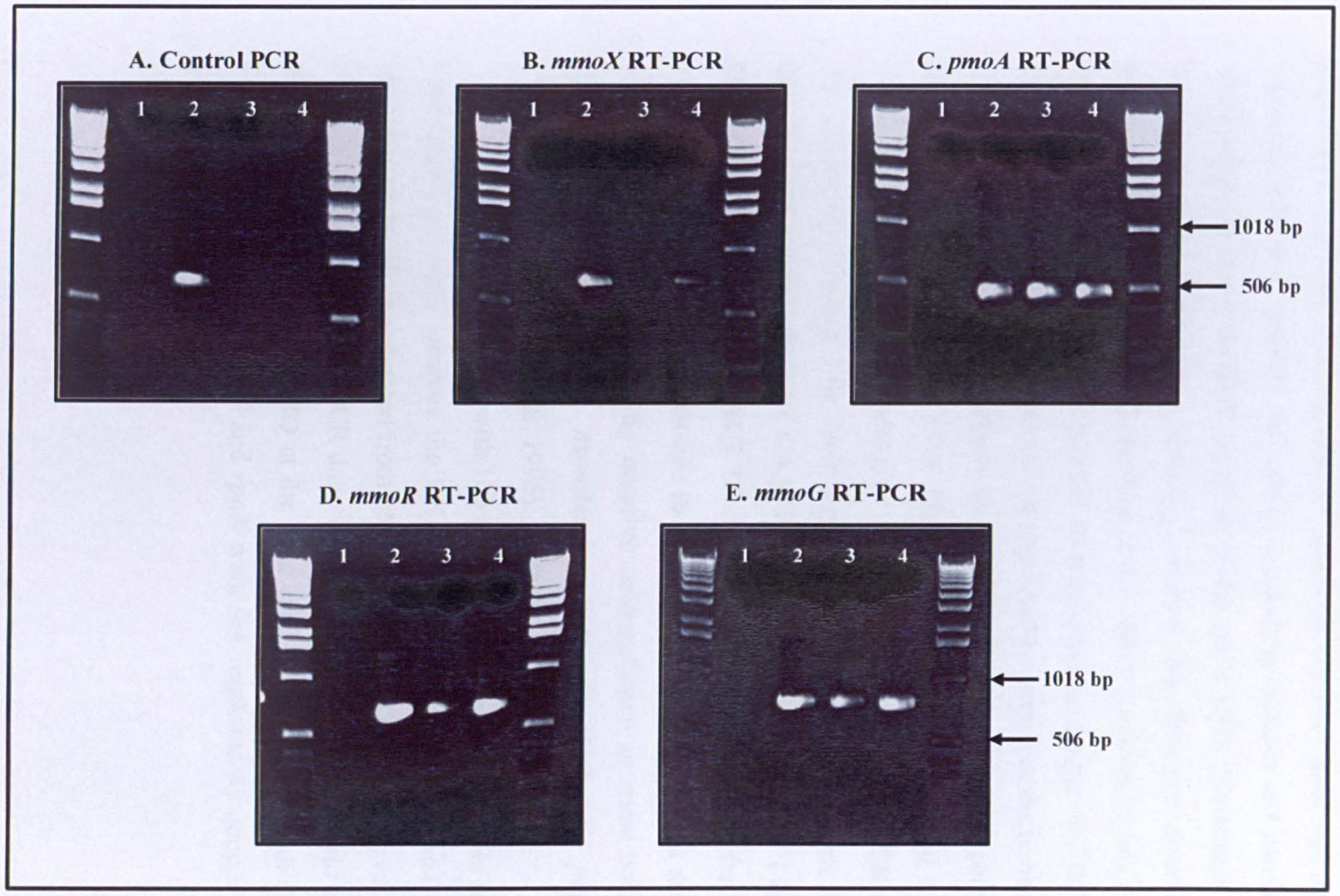

Figure 5.5 Transcriptional analysis of *mmoX*, *pmoA* and the sMMO regulatory genes *mmoR* and *mmoG* by RT-PCR. A. Control PCR on DNaseI treated RNA using *mmoX* primers. B-E. RT-PCR of *mmoX*, *pmoA*, *mmoR* and *mmoG* yielding 680 bp, 472 bp, 786 bp and 663 bp products, respectively. Lane contents: Lane 1, negative control; lane 2, positive control containing *Mc. capsulatus* genomic DNA; lane 3, RNA extracted from pMMO expressing (high copper) growth conditions; lane 4, RNA extracted from sMMO expressing (low copper) growth conditions.

The *mmoX* RT-PCR confirmed the MMO expressing condition of the RNA extracted since *mmoX* transcripts can only be detected in RNA when the copper-to-biomass ratio is low. The transcript for *pmoA* on the other hand was detected in RNA extracted from both pMMO and sMMO expressing cultures and confirmed the result obtained above using the BHR promoter probe, pMHA201. Mutational analysis of the regulatory genes, *mmoR* and *mmoG*, indicated that they are essential for sMMO expression (Csaki *et al.*, 2003; Stafford *et al.*, 2003), however, it was unclear whether these regulatory genes were subjected to regulation by copper. RT-PCR (Figure 5.5) indicated that *mmoR* and *mmoG* are not regulated by copper at the transcriptional level.

Total RNA extracted above for RT-PCR was also used to prepare RNA dot-blots as described in Section 2.7.3. The RNA dot-blots were probed with 16S rRNA, *mmoX*, *pmoA*, *mmoR*, *mmoG* and *rpoN* DNA probes, which were PCR amplified from *Mc. capsulatus* (Figure 5.6). Note: *rpoN* was PCR amplified from *Mc. capsulatus* using primers *rpoN*-F (5'-CAT CAA GCT GCT GCA GAT GTC-3') and *rpoN*-R (5'-CGG ACC AGA CGG ATC ACT TC-3'). The *rpoN* gene encoding for the σ^{54} regulon was used as a probe since although its expression is constitutive in many bacteria, it can be temporally regulated by negative autoregulation in some bacteria including *Acinetobacter calcoaceticus*, *Azotobacter vinelandii*, *Klebsiella pneumoniae* and *Pseudomonas putida* (Shingler, 1996).

The RNA blot probed with 16S rRNA assured that a consistent amount of RNA was loaded per slot, whereas the RNA dot-blot probed with *mmoX* confirmed the condition of the RNA extracted from pMMO and sMMO expressing cultures. The data obtained confirmed the RT-PCR data. Overall these data gave a clear insight into the differential regulation of MMO at the level of transcription. In addition, it indicated that the genes, *mmoR*, *mmoG* and *rpoN* were not regulated by copper at the level of transcription.

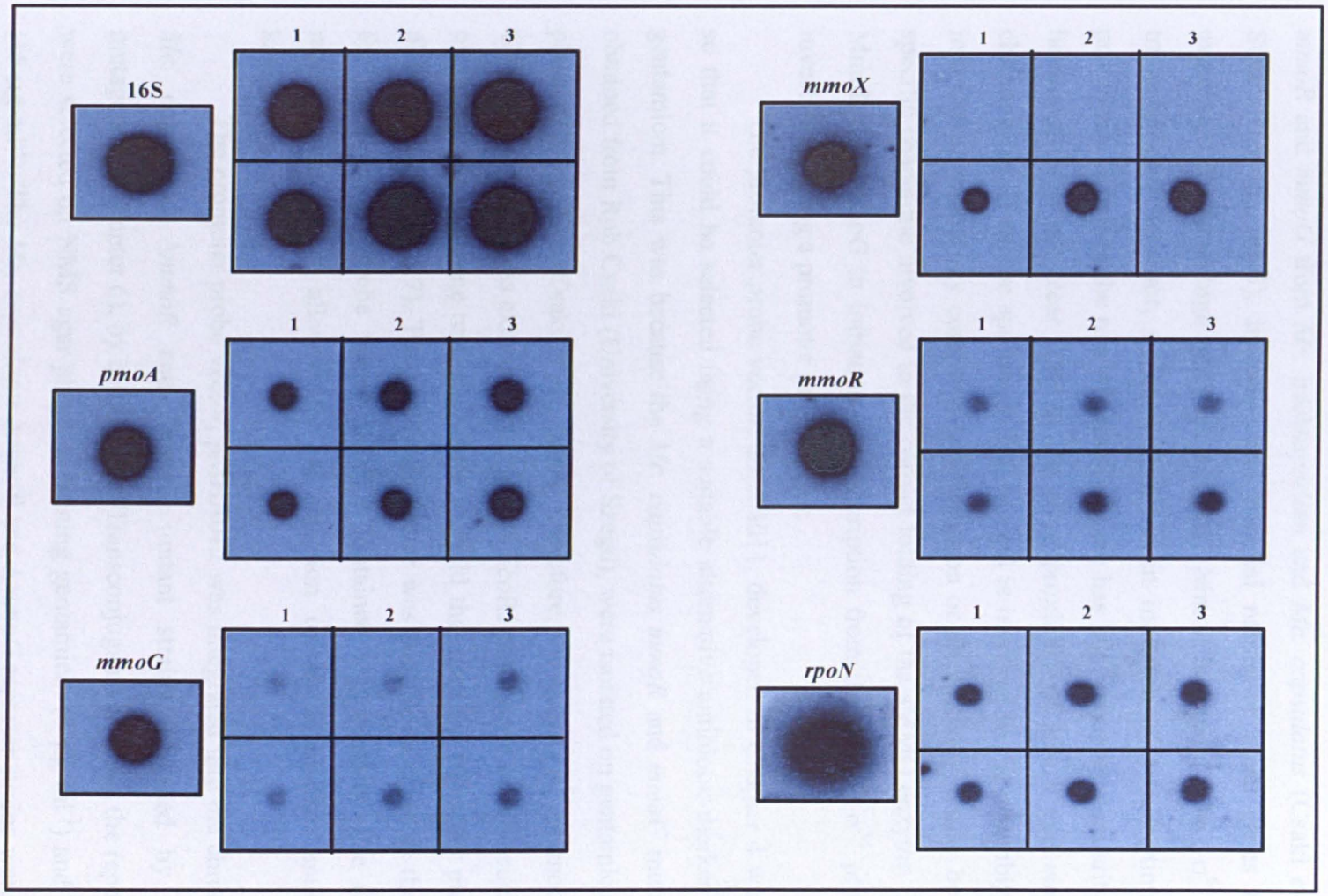

Figure 5.6 Transcriptional analysis of *mmoX*, *pmoA* and the sMMO regulatory genes *mmoR*, *mmoG* and *rpoN* by RNA dot-blot analysis. Each RNA dot-blot contains RNA extracted from pMMO (top row) and sMMO (bottom row) expressing *Mc. capsulatus* cultures, which were hybridised with a specific gene probe as indicated. Columns 1 – 3 contain approximately 0.5, 1.5 and 3.0 μg total RNA, respectively and approximately 6 ng of amplified gene probe was used as a positive control.

5.2.3 The roles of MmoR and MmoG in expression of sMMO

The initial cloning, sequencing and mutational analysis of the regulatory genes, *mmoR* and *mmoG* from *Ms. trichosporium* and *Mc. capsulatus* (Csaki *et al.*, 2003; Stafford *et al.*, 2003), indicated the essential nature of these genes for sMMO expression. Based on high sequence similarity, MmoR is thought to be a σ^{54}-dependent transcriptional activator, which is involved in initiation of transcription from σ^{54} promoters. However the role of MmoG, which has high sequence similarity to GroEL homologues, is not clear. Due to the close proximity of *mmoR* and *mmoG* in the chromosome, it can be speculated that MmoG is involved in the assembly of MmoR into a transcriptionally competent conformation or alternatively it may be a sMMO-specific chaperone involved in the correct folding of the sMMO enzyme. The role of MmoR and MmoG in initiation of transcription from the sMMO σ^{54} promoter was investigated using a promoter probe vector.

The promoter probe vector, pMHA011, developed in Chapter 4 was modified so that it could be selected using a suitable alternative antibiotic marker other than gentamicin. This was because the *Mc. capsulatus mmoR* and *mmoG* mutant strains, obtained from Rob Csaki (University of Szegd), were isolated on gentamicin to ensure plasmid integration (Csaki *et al.*, 2003). Therefore, the sMMO σ^{54} promoter fused to GFP in pMHA011 was excised with *Pst*I and *Eco*RI and ligated into the suicide vector, pK18mob via the same restriction sites to yield the modified promoter probe vector, pMHA012 (Figure 5.7). The pK18mob vector was suitable to serve as the backbone for the promoter probe vector since it contained a recognition site for plasmid mobilisation and it allowed for its selection on an alternative antibiotic (i.e. kanamycin).

The promoter probe vector, pMHA012 was integrated into the chromosome of *Mc. capsulatus* Δ*mmoR* and Δ*mmoG* mutant strains, obtained by transposon mutagenesis (Chapter 6), by conjugation. Transconjugants and thus the reporter strains were selected on NMS agar plates containing gentamicin (5 μg ml^{-1}) and kanamycin (15 μg ml^{-1}). The *Mc. capsulatus* Δ*mmoR* and Δ*mmoG* reporter strains were cultivated in a 5 L fermentor and cell-free extracts were prepared from exponentially growing cultures expressing pMMO and sMMO. However, after monitoring for GFP expression, no activity was detected in cell-free extracts prepared from pMMO and

sMMO expressing cultures. This indicated that both *mmoR* and *mmoG* were essential for transcription initiation from the sMMO σ^{54} promoter.

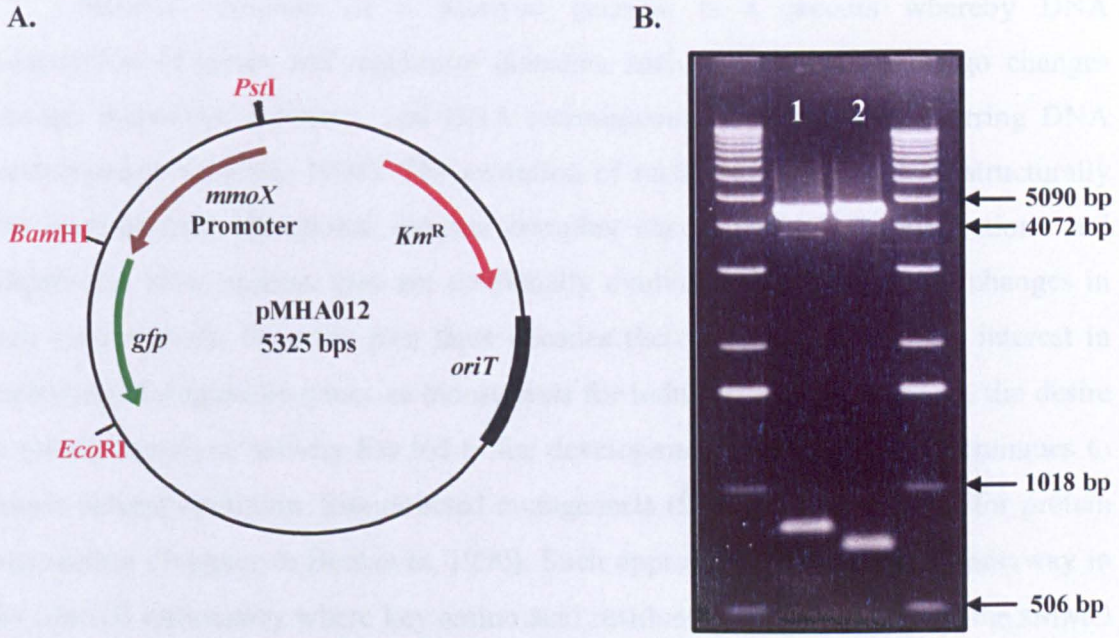

Figure 5.7 A. Physical map of the modified GFP promoter probe vector, pMHA012, containing sMMO σ^{54} promoter and the kanamycin resistance gene. B. Confirmation of the construction of pMHA012 by restriction digests analysis. Lane 1, *Pst*I and *Bam*HI digestion releasing the 821 bp *mmoX* promoter fragment. Lane 2, *Bam*HI and *Eco*RI digestion releasing the 754 bp *gfp* fragment.

5.3 Exploring the sMMO σ^{54} promoter and the attempted construction of a sMMO constitutive mutant

5.3.1 Introduction

Natural evolution of a bacterial genome is a process whereby DNA composition of genes and regulatory elements such as promoters undergo changes through mutations, deletions, and DNA rearrangement by naturally occurring DNA recombination (Arnold, 1998). The evolution of metabolic enzymes to a structurally and biochemically functional enzyme complex occurs over many generations and despite this slow process they are continually evolving to adapt to rapid changes in their environment. Over the past three decades there has been increasing interest in exploiting biological enzymes as biocatalysts for industrial use. In addition, the desire to enhance enzyme activity has led to the development of a number of techniques to mimic natural evolution. Site directed mutagenesis (SDM) is widely used for protein engineering (Wagner & Benkovic, 1990). Such approaches are currently underway in the Murrell Laboratory where key amino acid residues of the active site of the sMMO enzyme (i.e. α-subunit of the hydroxylase) are being mutated by SDM to alter substrate specificity. Error-prone PCR (EP-PCR) is an alternative approach for generating random point mutations and has the advantage that it is quick and easy to use. EP-PCR employs a low fidelity replication step to introduce random point mutations and using this technique mutants for example of lipases have been generated which have enhanced activity (Kohno *et al.*, 2001). In a similar fashion, EP-PCR has been used for detecting functional elements of proteins, for example the amino acid residues of σ^{54} in *E. coli* required for proper response to an activator were identified (Syed & Gralla, 1998).

Although directed evolution approaches are usually associated with modification of coding sequences followed by the selection of a enhanced protein activity, EP-PCR has been used in functional promoter analysis for identification of variant promoters with altered activity (Kagiya *et al.*, 2005; Remans *et al.*, 2005). Therefore, attempts were made to introduce random mutation in the sMMO σ^{54} promoter by EP-PCR, in the hope of identifying the upstream activating sequences, where it is believed the σ^{54} transcriptional activator, MmoR, binds and to generate promoter variants with altered activity. In addition, attempts were also made to

overcome the tight regulation of the sMMO σ^{54} promoter by replacing it with a strong constitutive promoter, such as the pMMO σ^{70} promoter, by 'knock-in' mutagenesis.

5.3.2 Construction of a modified LacZ reporter strain and error-prone PCR

The sMMO σ^{54} promoter was mapped by Csaki *et al.*, (2003) using a promoter probe vector and the region between -358 and -280 bp from the ATG of *mmoX* was thought to include the UAS involved in σ^{54}-dependent initiation of transcription (Csaki *et al.*, 2003). However, the reporter strains constructed in Chapter 4 included an 813 bp region of the sMMO σ^{54} promoter, which was more than necessary for transcription initiation. In order to reduce the region within which random point mutations were introduced by EP-PCR, a shorter region of the sMMO σ^{54} promoter was PCR-amplified using primer *Mc*-σ^{54}-F-*Pst*I (5'-CGA A**CT GCA G**TA CGG GAG ATC TGC-3') and *Mc*-σ^{54}-R-*Bam*HI (5'-CCT CCG TAA CAC A**GG ATCC** CAT GA-3') to yield a 505 bp fragment (Note: restriction sites were introduced within the primer, and as indicated by the primer name to facilitate cloning). The *lacZ* promoter probe vector, pMHA034, containing the larger sMMO σ^{54} promoter fragment was removed by digestion with *Pst*I and *Bam*HI and the shorter promoter fragment was digested and ligated into pMHA034 via the same restriction sites to yield the new *lacZ* promoter probe vector, pMHA036 (Figure 5.8A) (Note: The *lacZ* promoter probe vector was chosen for constructing additional *Mc. capsulatus* LacZ reporter strains since the activity of β-galactosidase could be detected with higher sensitivity, See Chapter 4).

The promoter probe vector, pMHA036 was conjugated into *Mc. capsulatus* and the *lacZ* reporter strain was selected on NMS agar plates containing gentamicin (5 μg ml^{-1}). The shorter promoter region was tested to ensure it was still functional. This was done by cultivating the reporter strain on NMS medium containing no added copper to induce the promoter and the β-galactosidase activity was monitored using the MUG assay (Figure 5.8B).

The construction of reporter strains containing mutated versions of sMMO σ^{54} promoter fused to LacZ would allow for the rapid screening of altered promoter activity by simply monitoring the β-galactosidase activity using the MUG assay. To this end, the σ^{54} promoter of sMMO was PCR-amplified to introduce errors with low and high frequency mutations using the GeneMorph II Random Mutagenesis Kit

(Strategene) and as described in Section 2.6.2. The mutated promoter fragments were digested with *Pst*I and *Bam*HI and ligated into pMHA034 after removing the larger sMMO σ^{54} promoter using the same restriction enzymes. The mutated promoter versions in pMHA036 were transferred to *E. coli* and a selection of the clones with low and high frequency mutations were analysed by sequencing (Figure 5.9A and B).

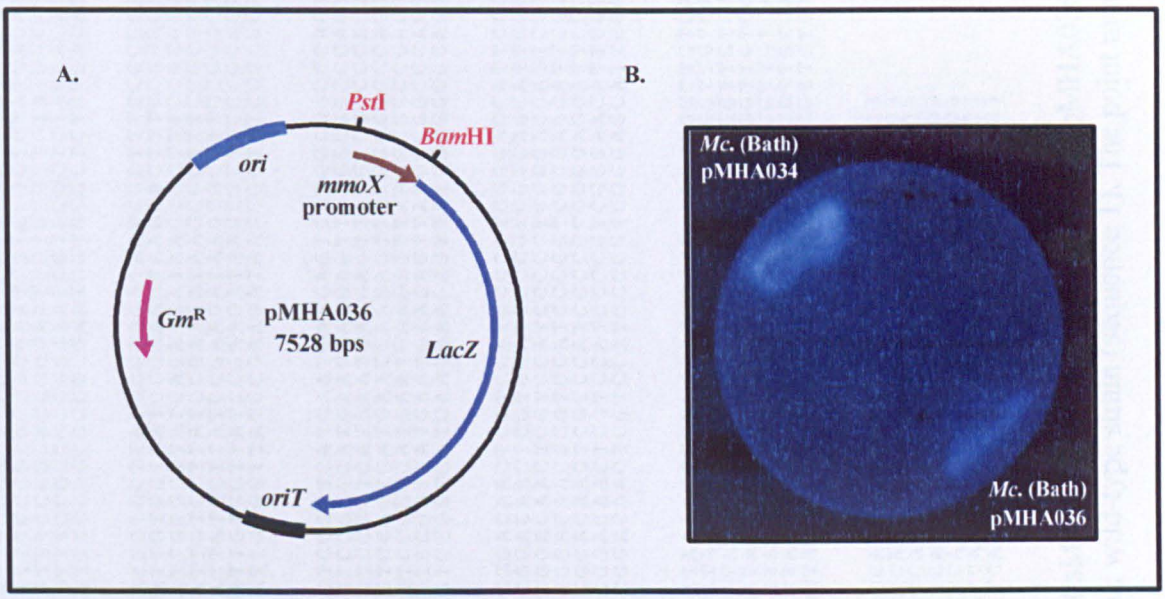

Figure 5.8 A. Map of the *lacZ* promoter probe vector, pMHA036, containing a shorter version of the sMMO σ^{54} promoter compared to that in pMHA034. B. Depicts β-galactosidase activity, using the MUG assay, of *Mc. capsulatus lacZ* reporter strain constructed by integration of pMHA036 into the chromosome. *Mc. capsulatus lacZ* reporter strain [pMHA034] was used as a positive control.

On average, 1.8 bp and 5.5 bp errors were introduced per promoter region amplified using low and high frequency mutation, respectively. This demonstrated that the rate of errors introduced can be carefully controlled using the method described in Section 2.6.2. It would have been interesting to combine a pool of *E. coli* S17.1 λ*pir* clones containing mutated versions of the sMMO σ^{54} promoter and conjugate them into *Mc. capsulatus*. However, due to time constraints this experiment was not finished.

Figure 5.9A Sequence alignment of six (L1-L6) mutated versions of sMMO σ^{54} promoter from *E. coli* [pMHA036] clones generated by low frequency mutation with sMMO σ^{54} promoter from *Mc. capsulatus* wild-type strain (sequence 1). The point mutations are highlighted in blue.

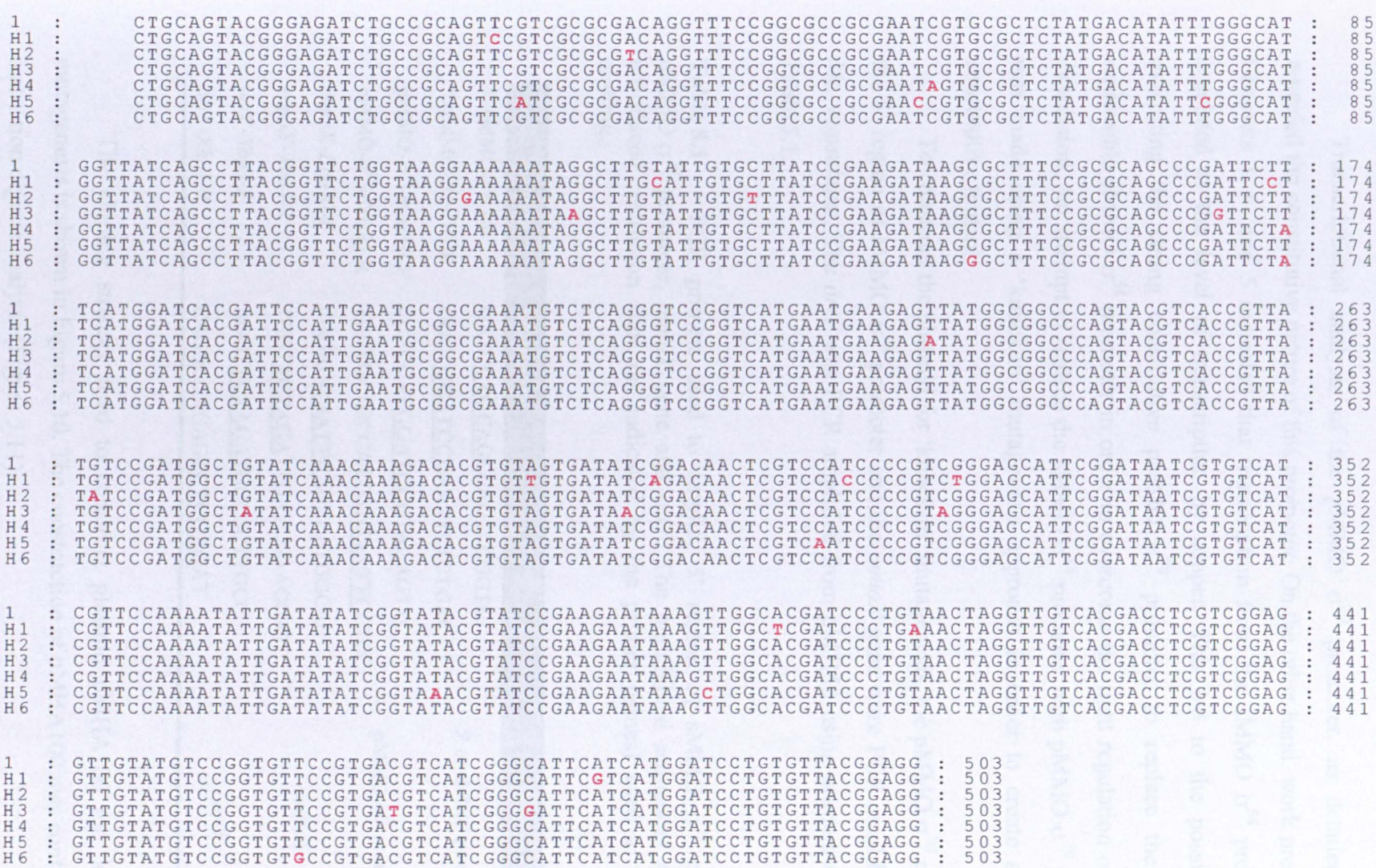

Figure 5.9B Sequence alignment of six (H1-H6) mutated versions of sMMO σ^{54} promoter from *E. coli* [pMHA036] clones generated by high frequency mutation with sMMO σ^{54} promoter from *Mc. capsulatus* wild-type strain (sequence 1). The point mutations are highlighted in red.

5.3.3 'Knock-in' mutagenesis

Transcriptional analysis of the pMMO σ^{70} promoter, as detailed above, revealed the constitutive nature of this promoter. On the other hand, work presented in Chapters 3, 4, and 5 indicated that transcription from the sMMO σ^{54} promoter is regulated at the level of transcription by copper. This led to the possibility of exploiting the strong constitutive pMMO σ^{70} promoter to replace the copper-repressible sMMO σ^{54} promoter in order to overcome the tight regulation of sMMO expression. An attempt to replace the sMMO σ^{54} promoter with pMMO σ^{70} promoter was made using a 'knock-in' mutagenesis approach in order to create a sMMO constitutive mutant.

To construct the plasmid for 'knock-in' mutagenesis, the pMMO σ^{70} promoter, the 5' region of sMMO σ^{54} promoter and the *mmoX* gene were PCR amplified from *Mc. capsulatus*. The *oriT* was PCR amplified from pMJ153 using primers shown in Table 5.1.

Table 5.1 PCR primers used to amplify 5' region of sMMO σ^{54} promoter, pMMO σ^{70} promoter, *mmoX* gene and *oriT*. The highlighted sequences show the introduced restriction sites (as indicated by the primer names) used to facilitate cloning.

Primers	Sequence	Gene
5'-sMMO-*Pst* I-F	GATC**CTGCAG**CCAGTCCGGTC	5' of sMMO promoter
5'-sMMO-*Bam* HI-R	GGCT**GGATCC**GAATTCTCTGAC	
pMMO-σ^{70}-*Bam* HI-F	CCACCAT**GGATCC**GTAGAGTTC	pMMO σ^{70} promoter
pMMO-σ^{70}-*Eco* RI-R	TGTTGCTGCCATTGT**GAATTC**CTCC	
mmoX-*Eco* RI-F	CGGAG**GAATTC**AGTAATGGCAC	*mmoX*
mmoX-*Xba* I-R	TGGT**TCTAGA**TTGATCGAACGATC	
oriT-*Xba* I-F	AGGT**TCTAGA**TGCCGCTTGCC	*oriT*
oriT-*Xho* I-R	CAGGCGAGAGC**CTCGAG**AT	

The cloning strategy used to construct plasmid pMHA100 for 'knock-in' mutagenesis is shown in Figure 5.10. The construction of pMHA100 was confirmed by restriction digests analysis (Figure 5.11).

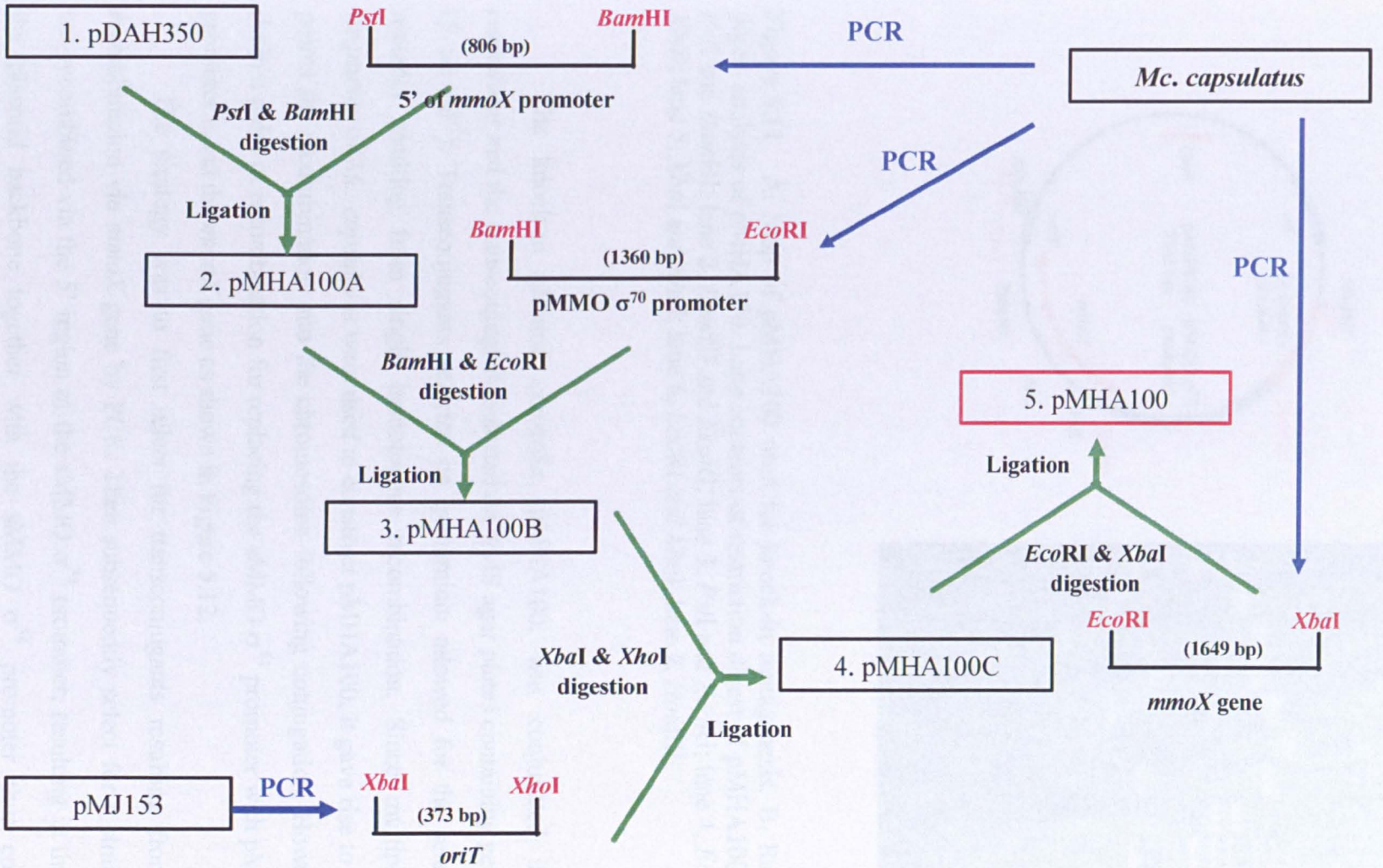

Figure 5.10 Schematic representation of the cloning strategy used for constructing pMHA100. The restriction sites highlighted in red were those introduced to facilitate cloning. The sizes of the DNA fragments following digestion are shown in brackets.

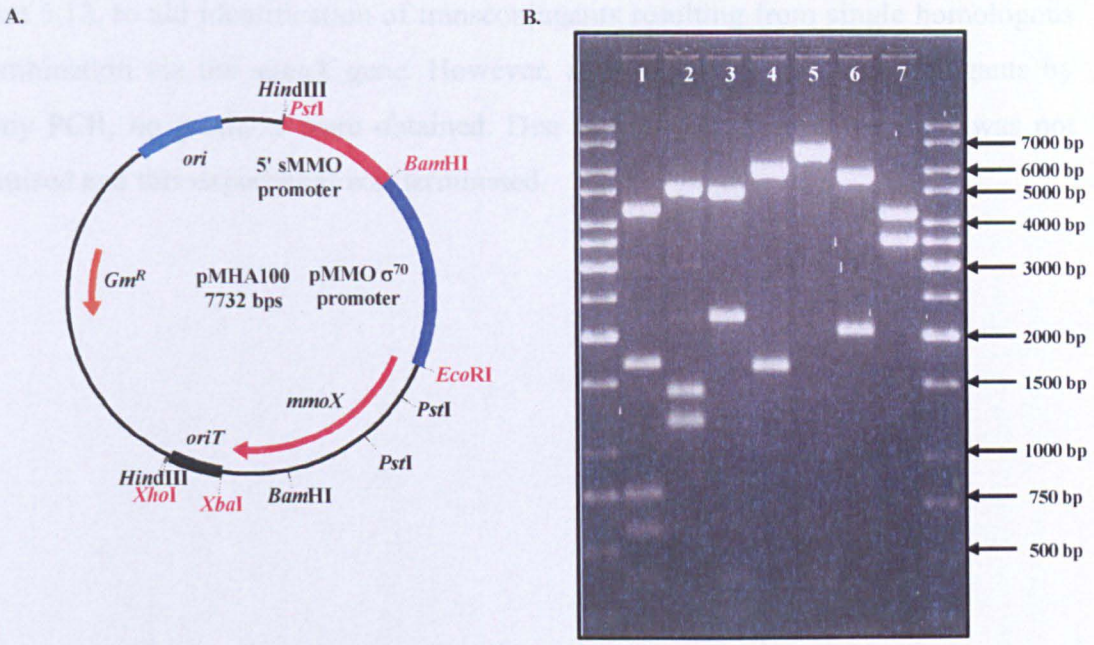

Figure 5.11 A. Map of pMHA100 used for knock-in mutagenesis. B. Restriction digest analysis of pMHA100. Lane contents of restriction digest of pMHA100: lane 1, *Pst*I and *Bam*HI; lane 2, *Bam*HI and *Eco*RI; lane 3, *Pst*I and *Eco*RI; lane 4, *Eco*RI and *Xba*I; lane 5, *Xba*I and *Xho*I; lane 6, *Eco*RI and *Xho*I; lane 7, *Hin*dIII.

The knock-in plasmid construct, pMHA100, was conjugated into *Mc. capsulatus* and the transconjugants selected on NMS agar plates containing gentamicin (5 μg ml^{-1}). Transconjugants selected on gentamicin allowed for the selection of mutants resulting from single homologous recombination. Since multiple DNA fragments of *Mc. capsulatus* were used to construct pMHA100, it gave rise to multiple points for recombination into the chromosome following conjugation. However, the desired point of recombination for replacing the sMMO σ^{54} promoter with pMMO σ^{70} promoter is via the *mmoX* gene as shown in Figure 5.12.

The strategy was to first select for transconjugants resulting from single recombination via *mmoX* gene by PCR. Then subsequently select for a strain which has recombined via the 5' region of the sMMO σ^{54} promoter, resulting in the loss of the plasmid backbone together with the sMMO σ^{54} promoter thus creating a gentamicin sensitive, sMMO constitutive *Mc. capsulatus* strain. PCR primers were designed based on the pMMO σ^{70} promoter (5'-GTT AGA GGC AGA ACC GAT AGG-3') and *mmoY* (5'-AGC TTC AGG AAG CGC CTT CA-3') as indicated in

Figure 5.12, to aid identification of transconjugants resulting from single homologous recombination via the *mmoX* gene. However, after screening six transconjugants by colony PCR, no products were obtained. Due to time constraints, the PCR was not optimised and this experiment was terminated.

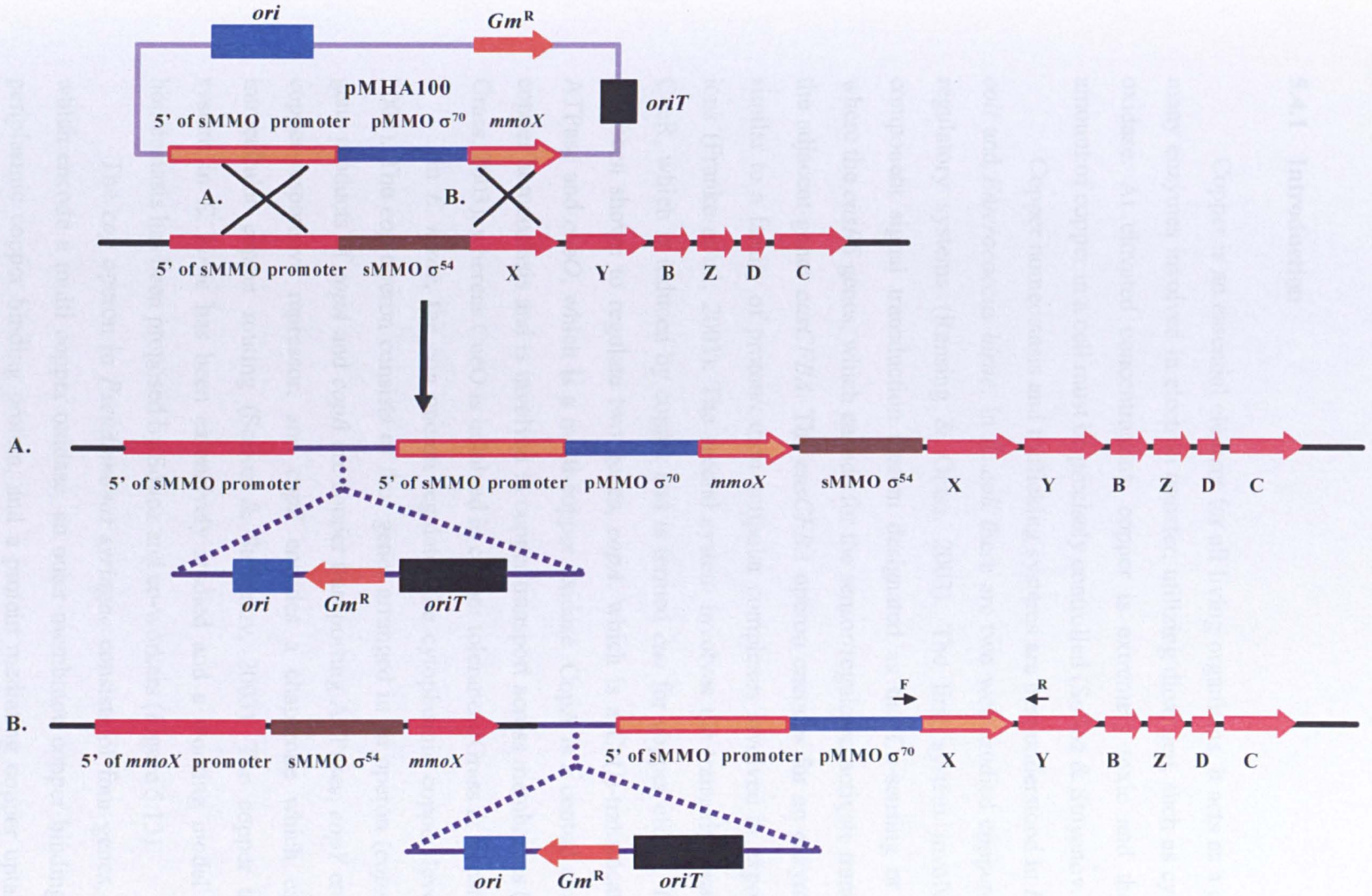

Figure 5.12 Schematic representation of the genotype of *Mc. capsulatus* chromosome following single homologous recombination of pMHA100 via A) 5' region of sMMO σ^{54} promoter and B) *mmoX*. Note: There is also a third point of recombination via the pMMO σ^{70} promoter (not shown). The primers used for screening transconjugants resulting from recombination via *mmoX* are indicated by black arrows.

5.4 Investigating copper transport by targeted mutagenesis

5.4.1 Introduction

Copper is an essential element for all living organisms. It acts as a cofactor for many enzymes involved in electron transfer, utilizing dioxygen, such as cytochrome *c* oxidase. At elevated concentrations, copper is extremely toxic and therefore the amount of copper in a cell must be precisely controlled (Solioz & Stoyanov, 2003).

Copper homeostasis and trafficking systems are best understood in *Escherichia coli* and *Enterococcus hirae*. In *E. coli* there are two well studied copper-responsive regulatory systems (Rensing & Grass, 2003). The first system involves a two-component signal transduction system designated as the Cu-sensing or *cus* locus, where the *cusRS* genes, which encode for the sensor/regulator, activate transcription of the adjacent genes *cusCFBA*. The *cusCFBA* operon encodes for an enzyme complex similar to a family of proton/cation antipolar complexes involved in export of metal ions (Franke *et al.*, 2003). The second system involves the transcriptional activator, CueR, which is induced by copper and is termed *cue* for copper efflux. This system has been shown to regulate two genes, *copA*, which is a Cu(I)-translocating P-type ATPase and *cueO*, which is a multi-copper oxidase. CopA is a central component in copper homeostasis and is involved in copper transport across membranes (Rensing & Grass, 2003), whereas CueO is involved in copper tolerance (Grass & Rensing, 2001).

In *E. hirae*, the *cop* operon regulates the cytoplasmic copper levels (Solioz, 2002). The *cop* operon consists of four genes arranged in an operon (*copABYZ*). The gene products of *copA* and *copB* are copper transporting ATPases, *copY* encodes for a copper-responsive repressor, and *copZ* encodes a chaperone which can catalyse intracellular copper routing (Solioz & Stoyanov, 2003). The copper transporting system in *E. hirae* has been extensively studied and a working model for copper homeostasis has been proposed by Solioz and co-workers (Figure 5.13).

The *cop* operon in *Pseudomonas syringae* consists of four genes, *copABCD*, which encode a multi copper oxidase, an outer membrane copper binding protein, a periplasmic copper binding protein, and a protein mediating copper uptake into the cytoplasm, respectively (Arnesano *et al.*, 2003). Like the *E. coli cus* system, *copABCD* is regulated by two-component signal transduction system encoded by *copRS*.

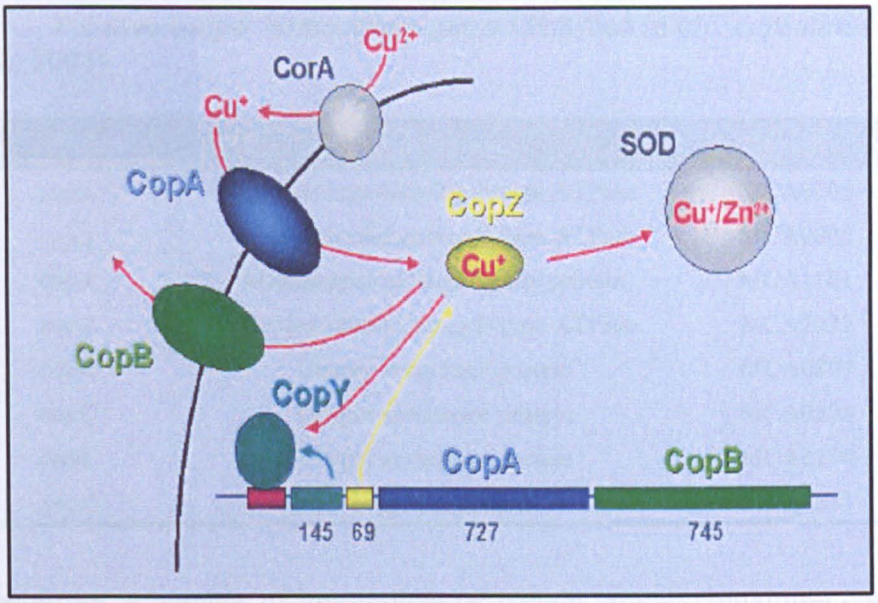

Figure 5.13 A working model for copper homeostasis in *E. hirae*. Extracellular copper is converted from Cu(II) to Cu(I) by the copper reductase, CorA, which is imported into the cell by CopA. The copper chaperone, CopZ picks up the copper and delivers it to intracellular copper-utilising enzymes such as superoxide dismutase (SOD). When there is excessive copper, CopZ delivers copper to CopB for secretion. In addition, it delivers copper to CopY, which binds the upstream P*cop* and represses transcription of the *cop* operon. Figure was obtained from http://www.ikp.unibe.ch/lab1/.

Mc. capsulatus is able to grow under a wide variety of copper conditions under which it regulates the expression of the metabolic MMO enzymes. Due to the central role copper plays in regulating MMO expression, it was crucial to understand the copper responsive regulatory systems in methanotrophs. The copper transporting systems in methanotrophs has been largely overlooked until the sequencing of the genome of *Mc. capsulatus* (Ward *et al.*, 2004). The genome sequence of *Mc. capsulatus* facilitated the identification of a number of homologues to *cus* genes found in *E. coli* and *cop* genes found in *E. hirae* and *P. syringae* (summarised in Table 5.2).

Table 5.2 Putative copper homeostasis genes identified in *Mc. capsulatus* genome (Ward *et al.*, 2004).

Gene (Putative)	Description	Gene No:
copA	Copper-translocating P-type ATPase	MCA0705
copA	Copper-translocating P-type ATPase	MCA0805
copA	Multicopper oxidase family protein	MCA1101
copA	Copper-translocating P-type ATPase	MCA2072
copC	Copper resistance protein	MCA0807
copC	Copper resistance protein	MCA0808
copC	Copper resistance protein	MCA2170
copZ	Copper chaperon	MCA0611

In addition, a number of low-molecular weight copper-containing compounds were previously identified (DiSpirito *et al.*, 1998). Since then the crystal structure of the copper-containing compound was resolved and renamed methanobactin, as it was found to be an analogous molecule to a extracellular iron-containing compounds such as siderophores (Kim *et al.*, 2004). Methanobactin was found to accumulate in the growth media of *Ms. trichosporium* and *Mc. capsulatus* when grown under copper-limited conditions. However, it was found to rapidly internalise when copper was provided. It was also found to co-purify with pMMO and the removal of methanobactin resulted in the loss of pMMO activity *in-vitro* (Kim *et al.*, 2004).

In light of these data it was hypothesised that methanobactin may serve as an unknown copper acquisition system in methanotrophs and may play a key role in stabilising the pMMO enzyme complex for active expression. Siderophores are small peptides synthesised non-ribosomally by genes encoding for non-ribosomal peptide synthetase (NRPS) or polyketide synthetase (PKS). From the genome of *Mc. capsulatus*, a number of putative genes encoding for NRPS has been identified, which may be involved in methanobactin biosynthesis (Table 5.3).

Table 5.3 Putative non-ribosomal peptide synthetase genes which might be involved in the biosynthesis of siderophores such as methanobactin

Gene No:	Description	Function (putative)
MCA1238	Polyketide synthase	Biosynthesis of methanobactin
MCA1883	Non-ribosomal peptide synthetase	
MCA2107	Non-ribosomal peptide synthetase, putative	

To take advantage of this information, a targeted mutagenesis approach was initiated in order to explore the copper transport systems and investigate whether methanobactin acted exclusively as an extracellular copper-sequestering agent or whether it had other *in-vivo* function, related to the delivery and insertion of copper ions to copper containing proteins like pMMO.

5.4.2 Mutagenesis of copper regulated targets

To construct knock-out mutants, two flanking DNA regions of MCA0705 (copper-translocating P-type ATPase) and MCA1883 (non-ribosomal peptide synthetase) were amplified by PCR and designated as product A and B, respectively. The primers used to amplify PCR products A and B are listed in Table 5.4.

Table 5.4 Primers used to amplify PCR products A and B from MCA0705 and MCA1883. Restriction sites were introduced via the primers to facilitate cloning (restriction sites are highlighted in bold and also underlined). [1]No restriction site was introduced in this primer since there was a *Sph*I site within the region amplified.

Primer	Sequence	PCR product
MCA0705AF-*Xba* I	5'-CGAAT**TCTAGA**TTGCGGTCGAAGG-3'	A
MCA0705AR-*Sph* I	5'-CCGTCA**GCATGC**ATTCGTCTACCG-3'	
MCA0705BF-*Sph* I	5'-CTTCATCG**GCATGC**CCATCCTC-3'	B
MCA0705BR-*Hin* dIII	5'-GGCGAC**AAGCTT**GTAACCGAAGG-3'	
MCA1883AF-*Xba* I	5'-CCTACA**TCTAGA**AGCTGGTCTTG-3'	A
MCA1883AR[1]	5'-CAATTCGAACTGGCGCTTGTC-3'	
MCA1883BF-*Sph* I	5'-TACCTCGCC**GCATGC**GTCGTTC-3'	B
MCA1883BR-*Hin* dIII	5'-CACTG**AAGCTT**GTCGCACAGGAAC-3'	

The plasmid constructs pMHA502 and pMHA504, for knocking out MCA1883 and MCA0705 respectively, were constructed using a strategy similar to that described in Section 3.5.3. Plasmids pMHA502 and pMHA504 were conjugated into *Mc. capsulatus* and mutants resulting from double homologous recombination were isolated through replica plating of the transconjugants onto NMS medium containing gentamicin (5 μg ml^{-1}) and then on medium containing gentamicin (5 μg ml^{-1}) and

kanamycin 15 μg ml^{-1}). Double cross-over mutants and thus the knock-out mutants were designated as *Mc. capsulatus* ΔMCA0705 and ΔMCA1883, respectively.

5.4.3 Characterisation of knock-out mutants

Initially the genotype of the mutant strains was confirmed to ensure the removal of the knock-out region. This was done by PCR using primers flanking the knock-out region. The primers used to confirm ΔMCA1883 strain were pMHA502KOF (5'-TGA AGA TCG ACC CTG CCA GAC-3') and pMHA502KOR (5'-AGG GAT TTC GTG CTC CGG TAG-3') and to confirm ΔMCA0705 strain primers pMHA504KOF (5'-ACG GCG AAG TCG TTC ACG G-3') and pMHA504KOR (5'-CCA GGC CAG ACC CAA TGT G-3') were used (Figure 5.14). (Note: The construction of ΔMCA1883 mutant strain involved the removal of a 1691 bp fragment from MCA1883 with the insertion of a 913 bp gentamicin resistance gene. ΔMCA0705 mutant strain involved the removal of a 576 bp region from MCA0705 with the insertion of a 913 bp fragment containing the gentamicin resistance gene).

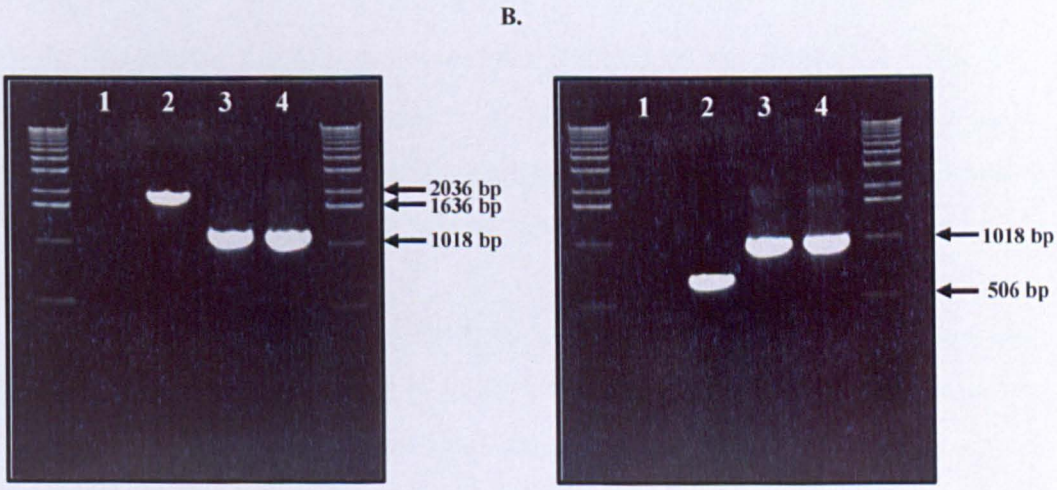

Figure 5.14 Confirmation of *Mc. capsulatus* ΔMCA1883 (Gel A) and ΔMCA0705 (Gel B) strains. Lane contents: lane 1, negative control; lane 2, PCR using DNA extracted from wild-type strain; lanes 3-4, DNA extracted from mutant strain. The differences in PCR products sizes obtained using DNA from wild-type and mutant strain indicated chromosomal knock-out of MCA1883 (Gel A) and MCA0705 (Gel B).

It was unclear what affect knocking out MCA1883 and MCA0705 had on the copper transport and MMO expression since no obvious phenotype could be detected

from both the mutant strains. On NMS agar plates, they grew as well as the wild-type strain. Further investigation needs to be done on the mutant strains in order to investigate the affect of the mutation on pMMO activity and their ability to express sMMO.

5.5 Discussion

5.5.1 Transcriptional regulation of MMO

The controversy surrounding the transcriptional regulation of the pMMO operon from a σ^{70} promoter by copper was resolved in this study. This was accomplished through the combination of a number of molecular techniques, which consistently showed the constitutive nature of the pMMO σ^{70} promoter.

RT-PCR data initially confirmed the constitutive nature of the pMMO σ^{70} promoter in *Mc. capsulatus* under high and low copper conditions. However, it did not give any indication into the relative abundance of the *pmoA* transcripts under the different copper conditions. The RNA dot-blotting data on the other hand suggested that the relative abundance of *pmoA* transcripts did not vary under high or low copper conditions. To support this result and to ensure that it was genuine, a number of controls were used. It is noteworthy that although RNA dot-blotting can not be used as an accurate quantification method for mRNA, it can give a good indication of the relative abundance of a particular transcript when appropriate controls are used. In addition, by indirectly monitoring *in-vivo* transcription of the pMMO σ^{70} promoter through the construction of a GFP reporter strain and measuring GFP expression under high and low copper conditions, it complemented the RT-PCR and RNA dot-blotting data. These data were consistent with the data obtained for *Ms. sporium* as discussed in Chapter 3.

The data presented in this study differed from those published from previous studies (Choi *et al.*, 2003; Stolyar *et al.*, 2001), which indicated that *pmoA* transcripts were significantly reduced under low copper conditions. Evidence exists which support the data presented in this study indicating that transcription from the pMMO σ^{70} promoter is constitutive and not repressed under low copper conditions, since sMMO-minus mutants were able to grow as well as the wild-type strain grown on NMS agar plates expressing pMMO under low copper conditions as shown in *Ms. sporium* (*mmoX* mutants; Chapter 3) and *Mc. capsulatus* (sMMO-minus transposon mutants; Chapter 6). However, it has been extensively shown in earlier expression studies performed on cell-extracts obtained from 15 L fermentor cultures that pMMO activity decreased with increasing sMMO activity when the copper-to-biomass ratio was low and visa versa (Prior, 1985). This is consistent with the observation of significantly

reduced pMMO polypeptides on SDS-PAGE when grown under sMMO expressing conditions (Choi *et al.*, 2003). In light of this information, it can be concluded that the transcription of the pMMO operon is not repressed under low copper concentrations and the expression of pMMO is regulated at a level post-transcription.

The regulation of sMMO expression is well established to be tightly controlled by copper from a σ^{54} promoter, as demonstrated in this study and in previous studies (Nielsen *et al.*, 1996; Nielsen *et al.*, 1997). To this end, it can be suggested that a unique 'copper-switch' regulates the expression of sMMO, which is regulated at the transcriptional level.

The exact mechanism regulating the sMMO transcriptional 'copper-switch' is not known. However in this study, it was demonstrated using a BHR promoter probe vector that MmoR and MmoG were both essential for transcription initiation from the σ^{54} promoter and further clarified the sMMO-minus phenotypes of these mutants constructed in *Mc. capsulatus* and *Ms. trichosporium* (Csaki *et al.*, 2003; Stafford *et al.*, 2003). It was not a surprise to learn that MmoR, a σ^{54}-dependent transcriptional activator, was involved in transcription initiation since σ^{54}-dependent transcription always requires a transcriptional activator (Reitzer & Schneider, 2001). However, it was interesting that MmoG, a GroEL homologue, was also essential in transcriptional activation and this may represent a potentially novel GroEL-dependent transcriptional activation mechanism and thus warrants further investigation.

The point at which copper exerts its ability to repress sMMO transcription was investigated by monitoring the transcription of the regulatory genes, *mmoR*, *mmoG* and *rpoN* under high and low copper conditions. As expected, the data clearly demonstrated that the *mmoR* and *rpoN* genes are transcribed under both high and low copper conditions. The transcription of *mmoG* under high and low copper conditions is interesting and is consistent with the hypothesis that it is required for transcription initiation and not for the post-translational assembly of sMMO polypeptides.

The exact mechanism by which the sMMO σ^{54} promoter is regulated is not known. σ^{54} transcriptional activators possess a sensory domain that can respond to stimuli in order to activate transcription (Shingler, 1996). Some regulators bind activating molecules directly, but there is no evidence of a copper-binding motif on the sensory domain of MmoR. As briefly discussed in Chapter 3, some subgroups of σ^{54} transcriptional activators operate as a response regulator for two-component regulatory

systems. In these systems a sensory histidine autokinase senses an environmental signal, which it subsequently transduces to the response protein (σ^{54} transcriptional activator) for its activation, which is achieved through a phosphotransfer transduction system (Figure 5.15) (Stock *et al.*, 1989). It is possible that the activity of MmoR is also regulated by phosphorylation where an unknown sensory histidine autokinase phosphorylates MmoR, which in turn activates the initiation of transcription when the copper-to-biomass ratio is low. Future experiments could involve mutating the sensory domain of MmoR to test the effect on sMMO regulation by copper.

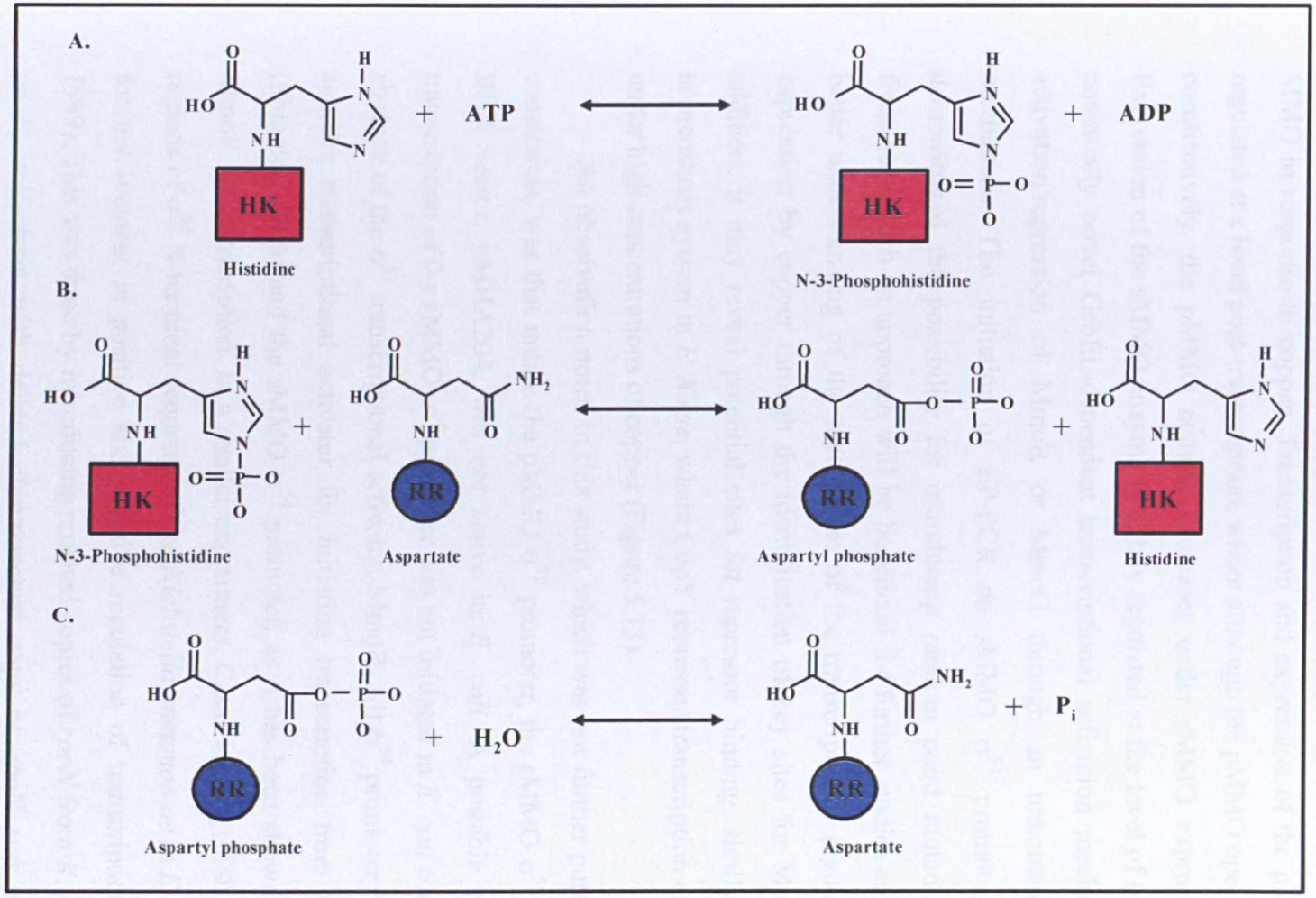

Figure 5.15 Phosphotransfer scheme in two-component transduction systems. A) Autophosphorylation: A phosphoryl group is transferred from ATP to a conserved histidine residue in the HK B) Phosphotransfer: The phosphoryl group from N-3-phosphohistidine side chain is transferred to a conserved aspartate residue in the RR C) Dephosphorylation: The phosphoryl group is finally transferred from the aspartyl phosphate side chain to aspartate and P_i via hydrolysis. Abbreviations: HK, Histidine Kinase; RR, Response regulator; ATP, adenosine triphosphate; ADP, adenosine diphosphate, P_i, inorganic phosphate.

5.5.2 Summary and future prospectives

In summary, it can be concluded that MMO expression is not regulated by a common 'copper-switch', which simply switches the concomitant on/off expression of MMO in response to copper. Transcription and expression of the pMMO enzyme is regulated at a level post-transcription, where although the pMMO operon is transcribed constitutively, the pMMO activity decreases under sMMO expressing conditions. Expression of the sMMO enzyme is tightly regulated at the level of transcription by a potentially novel GroEL-dependent transcriptional activation mechanism involving activation/repression of MmoR or MmoG through an unknown copper-related mechanism. The initiation of EP-PCR on sMMO σ^{54} promoter in this study demonstrated the possibility for introducing random point mutation with varying frequency. Such an approach will be beneficial for further studies since it can give a better understanding of the mechanism of the transcriptional regulation of sMMO expression by copper through the identification of key sites for MmoR binding. In addition, it may reveal potential sites for repressor binding, similar to the copper homeostasis system in *E. hirae*, where CopY represses transcription of the *cop* operon under high concentrations of copper (Figure 5.13).

An observation noted in this study, which was not further pursued due to time constraints, was that unlike the pMMO σ^{70} promoter, the sMMO σ^{54} promoter in the BHR vector, pMHA204, was not active in *E. coli*. A possible explanation why transcription of the sMMO σ^{54} promoter was not initiated in *E. coli* could be due to the absence of the σ^{54} transcriptional activator, MmoR. All σ^{54} promoter require a operon-specific transcriptional activator for initiating transcription from these promoters (Shingler, 1996) and the sMMO σ^{54} promoter, as it has been shown above, requires MmoR for transcription. In a similar experiment, Casaz *et al.*, (1999) investigated the regions of σ^{54} N-terminal sequences from *Klebsiella pneumoniae* in *E. coli* by EP-PCR for involvement in positive and negative regulation of transcription (Casaz *et al.*, 1999). This was done by introducing mutated copies of *rpoN* from *K. pneumoniae* into an *E. coli* strain with deleted chromosomal copy of *rpoN* and measured the β-galactosidase activity which was placed under the control of *K. pneumoniae nifH* σ^{54} promoter. In order for this system to work in *E. coli*, they reported the requirement of expression of the σ^{54} transcriptional activator, NifA. Based on this study, it is possible

that sMMO σ^{54} promoter in *E. coli*, when supplemented with the transcriptional regulators, *mmoR* and *mmoG* on a compatible plasmid is likely to be active. This would allow mutagenesis studies on the sMMO σ^{54} promoter, MmoR, MmoG and RpoN to be carried out in *E. coli* which could be used to unravel the molecular mechanism of transcriptional regulation of the sMMO.

The attempted knock-in mutagenesis approach for constructing a sMMO constitutive mutant had to be halted due to lack of time. However, following the conjugation of pMHA100 in *Mc. capsulatus*, six transconjugants were screened for single homologous recombination via *mmoX* by colony PCR. Unfortunately, no PCR products were obtained. There are a number of reasons why no products were obtained. Colony PCR often can be inhibited by cell debris and requires optimisation or it could be that none of the transconjugants screened were a result of recombination via the *mmoX* gene. These problems can be overcome in future studies by screening more transconjugants using purified genomic DNA as template for PCR. Since the plasmid for creating such mutant has been already constructed in this study, it would be beneficial for future studies to use such a system for isolating a constitutive sMMO mutant for expressing site-directed mutants of sMMO hydroxylase generated in this lab.

It is well established that copper plays a central role in methane oxidation in methanotrophs and for this reason, a targeted mutagenesis approach was taken to characterise some of the copper genes identified in the *Mc. capsulatus* genome. Two knock-out mutants were generated in this study, one in a copper-translocating P-type ATPase gene involved in copper transport and the other in a NRPS, which might be involved in the biosynthesis of siderophore such as methanobactin. Unfortunately no phenotypes for these mutants were observed. However it cannot be ruled out that they are not involved in copper transport since there seems to be multiple copper transport systems in methanotrophs. For example, there are several genes encoding for copper-translocating P-type ATPase and a mutation in one copy may be compensated for by another. In order to investigate the copper transporting system in methanotrophs, it may require multiple mutations in copper transporting genes. One of the factors limiting genetic manipulations in methanotrophs is the lack of suitable selectable antibiotic markers. In future studies these problems can be circumvented by using a Cre-*loxP* system (Figure 5.16) where the antibiotic marker could be recycled to create

multiple gene disruptions as shown in *M. extorquens* AM1 (Marx & Lidstrom, 2004) and *Dictyostelium discoidem* (Faix *et al.*, 2004).

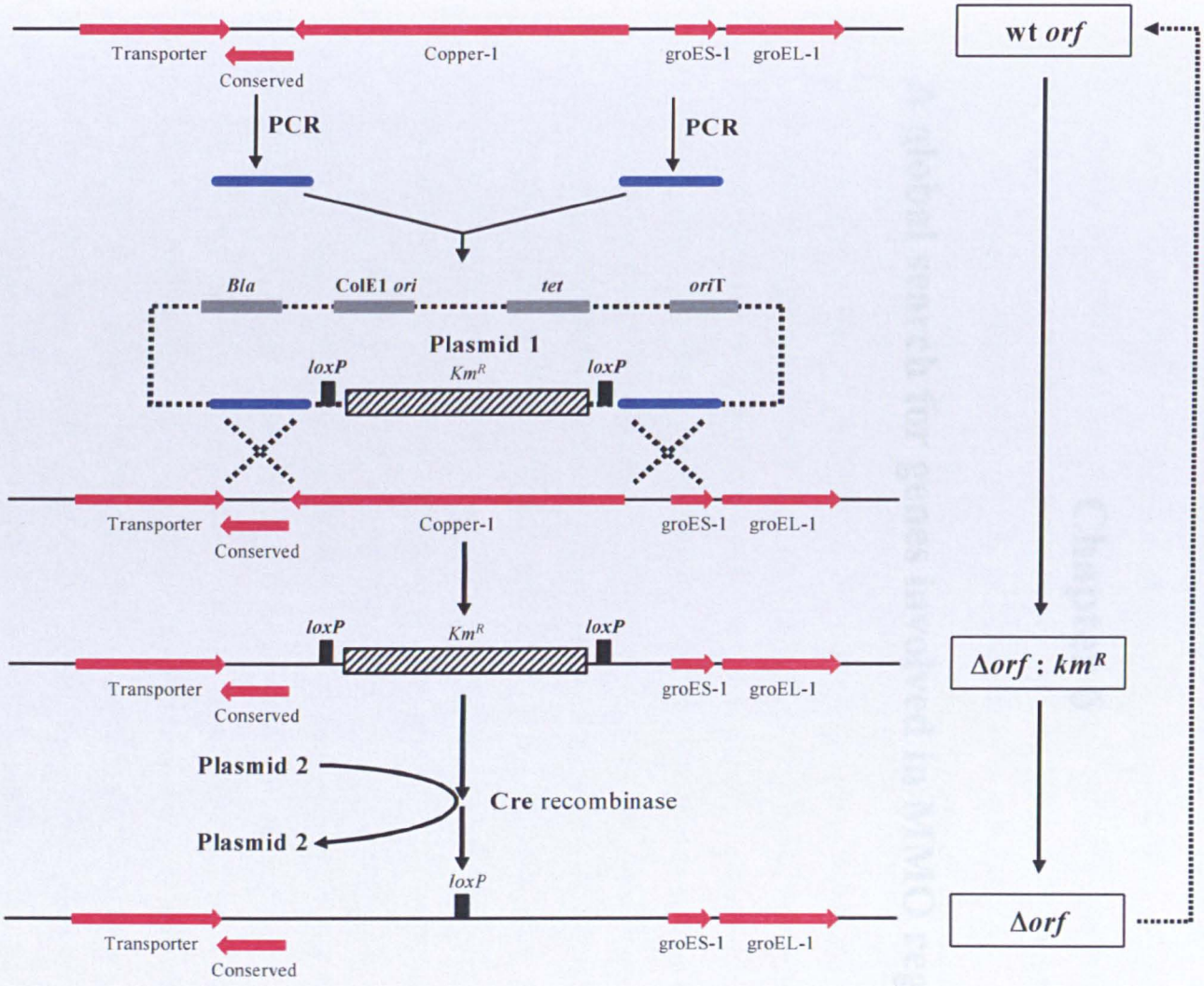

Figure 5.16 *Cre-lox* system for antibiotic marker recycling in Gram-negative bacteria. An example knock-out mutagenesis strategy of a copper gene in *Mc. capsulatus*. Plasmid 1 is used to create a knock-out mutant by marker-exchange mutagenesis, where the selectable km^R gene is flanked by *loxP*, which are sites for recombination. Plasmid 2 contains Cre recombinase and when introduced in the mutant strain, it will remove the Km^R cassette through intramolecular recombination and leaves a kanamycin-sensitive mutant strain.

Chapter 6

A global search for genes involved in MMO regulation

6.1 Introduction

The rapid explosion in whole genome sequencing of a number of microbial organisms over the past 10 years has generated unprecedented amounts of sequence information. To date there are 343 complete microbial genome sequences available at NCBI alone and a further 586 microbial genome sequencing projects that are currently in progress (May, 2006). In addition, in recent years there has been increasing interest in carrying out genetic analysis of bacteria other than *E. coli*. One group of organisms that has received considerable interest over the past 30 years are methanotrophs, such as *Mc. capsulatus* due to their ecological significance in processes such as methane cycling and their vast potential for biotechnological applications (See Chapter 1).

The first genome sequence of a methane-oxidising bacterium, *Mc. capsulatus* (NCBI Accession No: NC_002977) has led to the direct identification of a number of targets for mutagenesis for investigating the regulation of MMO on a broader scale. The initial decision to capitalise on the genome sequence information was put into practice using a hypothesis driven marker-exchange mutagenesis approach. However, following the initial construction of knock-out mutants in genes encoding a non-ribosomal peptide synthetase and a copper-translocating P-type ATPase (See Chapter 5) it was quickly realised that this method was not suitable since it was time consuming and technically involved. Furthermore, the detailed analysis of the MMO regulation at the transcriptional level gave further insights into the complexity of the molecular regulation of the MMO operons and skewed our perception of the 'copper-switch' as an on-off switch from pMMO to sMMO expression (Chapter 5). In light of this information, it was clearly necessary to proceed with an experimental approach which can be used in a high-throughput fashion. In addition, it should also allow the retrieval of information on global gene regulation network at the transcriptional (mRNA) level or at the translational (protein) level under different environmental conditions. This level of study is commonly referred to as 'Functional Genomics' since it involves utilising the genome sequence to study genes and the function of the resulting proteins (Oliver *et al.*, 2002). The techniques currently available for functional genomic studies, which fulfil these experimental criteria to some extent include microarray technology, proteomics approaches utilising 2-D gel electrophoresis (2-DE) for protein separation coupled with mass spectrometry (MS) for protein identification and a genetic approach utilising transposon such as Tn*5* for

generating random mutants. However, none of these techniques are perfect and without pitfalls.

Microarray technology has rapidly evolved over the past 5 years as a powerful tool for understanding the regulation of gene expression in bacteria (Rhodius & LaRossa, 2003). In recent years there have been numerous publications on the use of microarrays for studying genome-wide expression profiles of 'regulons', which are sets of transcription units controlled by a single regulatory protein (Britton *et al.*, 2002; Manganelli *et al.*, 2001). Microarray methods for determining mRNA levels are predominantly based on cDNA microarrays, where DNA targets are covalently bound to a glass slide and cDNAs labelled with flurophores are used as molecular probes for hybridisation with the array (Oliver *et al.*, 2002). Using this system, the transcriptional profile of thousands of genes can be represented on a single slide. The major problems limiting wide-spread use of cDNA arrays are its high expense and time intensiveness to set up a well organised array experiment. In addition, specificity and sensitivity are also a problem and require careful experimental consideration (Cook & Sayler, 2003). Microarray technology is ultimately based on mRNA and recognising the fact that isolated mRNA does not necessarily reflect the situation *in-situ* can be problematic when trying to address certain question. For example the mRNA transcripts of *pmoA*, as briefly discussed in Chapter 3 and in more detail in Chapter 5, can not be approximated to protein expression since expression is regulated at a post-transcriptional level. Due to these reasons, a microarray approach to survey the *Mc. capsulatus* genome for MMO regulatory genes was not pursued in this study. However, recent studies carried out in Harald B. Jensen's group (University of Bergen) are focusing on the development and optimisation of a microarray protocol for surveying the genome-wide transcriptional regulation of gene expression in *Mc. capsulatus* under different copper conditions. The initial analysis of the preliminary data looks very promising (Live J. Bruseth, personal communication) and will be an excellent platform for complementing the data presented in Chapter 5.

Over the past 10 years the field of proteomics for studying the global protein expression profile in two different states has rapidly developed in parallel with the DNA sequencing technology (Griffin *et al.*, 2001). Proteomics combines the traditional protein separation technique of 2-DE and MS technology in protein detection and measurement as a powerful tool for functional genomic analysis of a proteome at a particular cell state. The rapid development of 2-DE and MS technology

has allowed for the automation of protein separation and identification and has subsequently evolved into a versatile high-throughput method for the simultaneous identification of hundreds of proteins in a single operation (Andersen & Mann, 2000; Zhu *et al.*, 2003). Despite the advances in proteomics, there are still major limitations especially with regards to protein separation using 2-DE. These limitations include the bias for highly abundant proteins with the exclusion of low-abundance or very low or very high molecular weight proteins. Furthermore, protein separation using 2-DE is extremely difficult to automate (Gygi *et al.*, 2000; Patton *et al.*, 2002). These limitations in protein separation were circumvented with the development of an experimental strategy for quantitative proteomics using a chemical reagent termed isotope-coded affinity tags (ICAT) (Aebersold, 2003). Although the ICAT approach to proteomics resolved the problem of selective exclusion of some classes of proteins, since it did not involve 2-DE for protein separation, it had the inherent limitation of not being able to identify peptides lacking a cysteinyl residue. This can be a major problem when analysing the proteome of a microbe since >25 % of the proteins lack cysteine (Aebersold, 2003). Despite their limitations, both of these proteomics approaches are powerful tools for analysing global protein expression profile and have been used to survey the proteome of *Mc. capsulatus* (Fjellbirkeland *et al.*, 1997; Kao *et al.*, 2004). Therefore, a proteomics approach for analysing the proteome of *Mc. capsulatus* was not considered as part of this study since it had already been done. However, the identification and analysis of an outer membrane protein termed MopE by Fjellbirkeland *et al*, (1997) using the 2-DE/MS approach, revealed the responsiveness of MopE to copper (Karlsen *et al.*, 2003). In light of these data, the initial aim of this study was to analyse an *orf* (MCA2590) located immediately upstream of *mopE* and investigate whether it played a role in MMO expression.

Bacterial transposons are DNA segments often termed 'mobile genetic elements' that can move from one location on a DNA sequence and insert themselves into another and in doing so generate mutations by means of insertional inactivation in a randomised fashion (Berg & Berg, 1983). A number of different families of transposons have been discovered to date, however, since the initial discovery of the Tn*5* transposon, several sophisticated vectors carrying antibiotic resistance genes based on Tn*5* have been developed, which allow the introduction of the transposable element into the genome of different bacteria (Simon *et al.*, 1983; Simon, 1984).

The Tn*5* transposon has now been around for several decades and since its initial discovery it has proved to be an extremely powerful tool in bacterial genetics. Even prior to the genomic era, transposon mutagenesis experiments greatly facilitated the global search for genes involved in different biological processes including the genes encoding for bioluminescence in *Vibrio fischeri* and naphthalene degradation in *Klebsiella pneumoniae* (de Bruijn & Lupski, 1984; Rossignol *et al.*, 2001). One of the major advantages of this approach, making it a very powerful tool for surveying the genome of *Mc. capsulatus* for genes involved in MMO regulation, over the previous two approaches described above, is that transposon mutagenesis usually involves gene disruption. Since one of the definitive ways of linking a gene encoding a particular protein to its function is by mutagenesis, transposon mutagenesis was the strategy of choice in this study to address the question of MMO regulation by copper. It is noteworthy, since the subsequent analysis of the location of the transposon required the cloning of the transposon together with the flanking DNA sequence, *Mc. capsulatus* was used for this study since the annotated genome sequence was available.

In order to take advantage of this approach, a phenotypic screen for the detection of the desired class of mutants is absolutely essential and this is often the major factor limiting its widespread use since the phenotype of a gene is often not known. Furthermore, on the occasions when the phenotype of a mutant is known, it is not always possible to develop a rapid screening system. In light of this information, the development of a transposon mutagenesis protocol and the development and analysis of a high-throughput genetic screening method for the global search of the genes involved in MMO regulation in *Mc. capsulatus*, formed the main aims of this study.

6.2 Characterisation of the surface associated protein, SapE

6.2.1 Introduction and rationale of this study

The initial work carried out by Fjellbirkeland *et al*, (1997) on the global survey of the outer membrane proteins in *Mc. capsulatus* via a proteomics approach, led to the identification of five major outer membrane proteins, which were designated as MopA-E (Fjellbirkeland *et al.*, 1997). The protein designated as MopE, a 66 kDa protein, was of particular interest since it was shown to be localised on the cell surface and periphery of the cell. In addition, the expression of MopE was shown to be regulated by copper and was found to be highest under low copper conditions (i.e. sMMO expressing conditions) (Karlsen *et al.*, 2003). Furthermore, under low copper condition, a 46 kDa protein was secreted into the growth medium, which was shown to be the C-terminal part of MopE and was designated as MopE*.

The sequencing of the *Mc. capsulatus* genome and the subsequent bioinformatics analysis revealed the presence of an *orf* (MCA2590), which was designated as *sapE* (surface assocated protein) due to its cell surface localisation. The *sapE* gene was located 5' of the *mopE* gene. Further work demonstrated the responsiveness of SapE expression with respect to copper in a similar fashion to that of MopE described above (Kindingstad, 2003). The genetic arrangement of *mopE* and *sapE* in the chromosome (Figure 6.1) and their apparent co-regulation under low copper conditions coupled with the fact that MopE was involved in binding copper (Anne Fjellbirkeland, personal communication) warranted further investigation, especially with regards to MMO regulation.

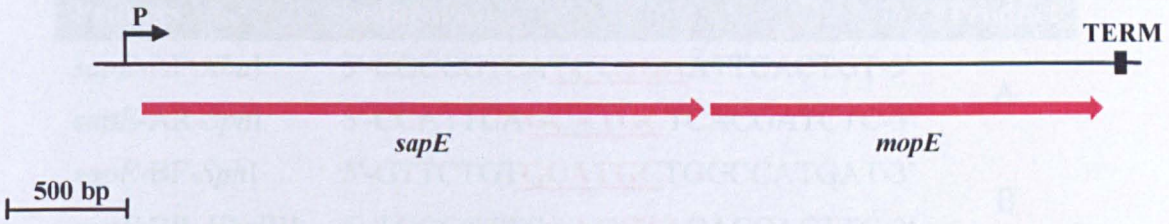

Figure 6.1 The arrangement of the putative *sapE/mopE* operon. The bioinformatics analysis used to predict the putative promoter (P) and transcription termination site (TERM) were described by Karlsen *et al.*, (2005).

The hypotheses of this study were based on the following experimental observations. MopE was found to be easily degraded under low copper conditions to MopE*, however, this degradation was prevented in the presence of protease inhibitors (Anne Fjellbirkeland, personal communication). Therefore it was speculated, due to the close correlation of SapE and MopE expression, that SapE may be involved in the proteolysis of MopE. In addition, as mentioned above, MopE was shown to bind copper and thus one of the working hypotheses was that SapE and MopE are involved in copper transport to the cell and therefore may be involved in the regulation of MMO expression. To this end, in a joint collaboration with Dr Anne Fjellbirkeland, phenotypic characterisation of MopE (Anne Fjellbirkeland, University of Bergen) and SapE (This study) mutants were initiated in this study.

6.2.2 Construction of *Mc. capsulatus* Δ*sapE* mutant strain

The construction of a mutant strain of *Mc. capsulatus* defective in its ability to express SapE will allow the above hypotheses to be thoroughly tested. The strategy used to knock-out a DNA portion internal to the *sapE* gene was similar to the strategy used to construct knock-out mutants of *mmoX* described in Chapter 3 and NRPS and copper targets described in Chapter 5. Two internal DNA portions of *sapE*, designated as product A (2165 bp) and B (1611 bp) were PCR amplified using primers listed in Table 6.1.

Table 6.1 PCR primers used to amplify internal DNA portions of the *sapE* gene. The sequences highlighted in red and in bold face indicate the in-frame introduction of restriction sites, as indicated by primer names, to facilitate cloning.

Primers	Sequence	Product
sapE-AF-*Xba*I	5'-CGCCGTCA**TCTAGA**ATTCACTGT-3'	A
sapE-AR-*Sph*I	5'-CCATTCA**GCATGC**TCACGATCTC-3'	
sapE-BF-*Sph*I	5'-GTTCTGT**GCATGC**TGGCCATGAT-3'	B
sapE-BR-*Hin*dIII	5'-TGCCGTTG**AAGCTT**GACCACTTG-3'	

The PCR products A and B were cloned into the suicide vector, pK18mob via *Xba*I and *Hin*dIII sites to yield the intermediate construct pMHA506.1. A 913 bp *Sph*I

fragment containing a Gm^R gene was obtained from p34S-Gm, which was subsequently cloned into the *Sph*I site located between products A and B in pMHA506.1 to give the final construct, pMHA506 (Figure 6.2). Prior to conjugation, the construction of the suicide vector, pMHA506 was confirmed by restriction digest analysis in addition to sequencing using the universal M13 forward and reverse primers.

Electrocompetent cells of *E. coli* S17.1 λ*pir* were transformed with pMHA506 and the resulting transformants were used as a donor strain for conjugation with *Mc. capsulatus* using methods described in Section 2.5.1. The respective transconjugants were initially selected on NMS agar plates containing gentamicin (5 μg ml^{-1}), which selected both single and double homologous recombinants. These were then subsequently selected on NMS agar plates containing gentamicin (5 μg ml^{-1}) and kanamycin (15 μg ml^{-1}) to separate the single recombinants from the double recombinants. Note: Only single recombinants will be able to grow on NMS agar plates containing kanamycin (15 μg ml^{-1}), since transconjugants resulting from double homologous recombination lose the plasmid backbone containing the kanamycin resistance gene and thus become sensitive to kanamycin.

From the 68 transconjugants screened, only 6 were found to be a result of double homologous recombination, since they were unable to grow on NMS agar plates containing kanamycin (Figure 6.3). Isolation of the double homologous recombinants yielded a *Mc. capsulatus* Δ*sapE* mutant strain, with an internal portion of *sapE* gene replaced with a gentamicin resistance gene.

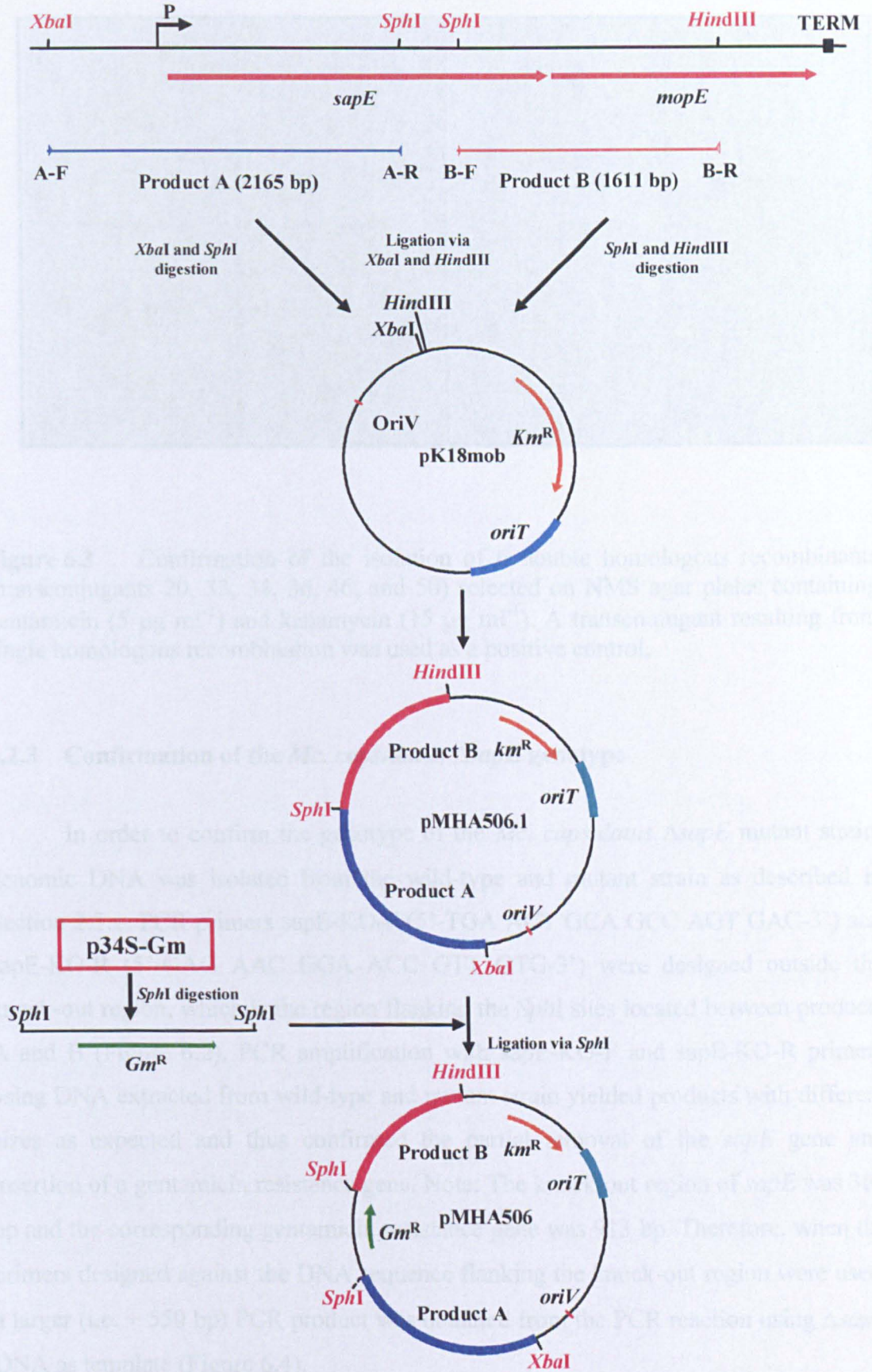

Figure 6.2 Schematic representations of the cloning steps involved in the construction of the suicide vector, pMHA506. Restriction sites highlighted in red are those introduced during PCR amplification and are involved in cloning.

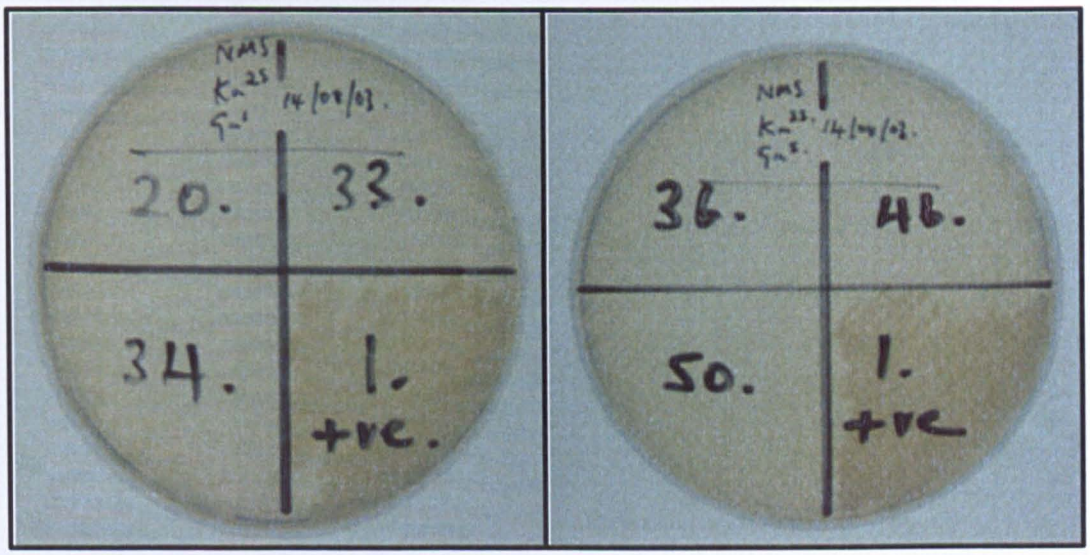

Figure 6.3 Confirmation of the isolation of 6 double homologous recombinants (transconjugants 20, 33, 34, 36, 46, and 50) selected on NMS agar plates containing gentamicin (5 μg ml^{-1}) and kanamycin (15 μg ml^{-1}). A transconjugant resulting from single homologous recombination was used as a positive control.

6.2.3 Confirmation of the *Mc. capsulatus* Δ*sapE* genotype

In order to confirm the genotype of the *Mc. capsulatus* Δ*sapE* mutant strain, genomic DNA was isolated from the wild-type and mutant strain as described in Section 2.3.1. PCR primers sapE-KO-F (5'-TGA ACT GCA GCC AGT GAC-3') and sapE-KO-R (5'-GAG AAC GGA ACC GTT GTG-3') were designed outside the knock-out region, which is the region flanking the *Sph*I sites located between products A and B (Figure 6.2). PCR amplification with sapE-KO-F and sapE-KO-R primers using DNA extracted from wild-type and mutant strain yielded products with different sizes as expected and thus confirmed the partial removal of the *sapE* gene and insertion of a gentamicin resistance gene. Note: The knock-out region of *sapE* was 363 bp and the corresponding gentamicin resistance gene was 913 bp. Therefore, when the primers designed against the DNA sequence flanking the knock-out region were used, a larger (i.e. + 550 bp) PCR product was obtained from the PCR reaction using Δ*sapE* DNA as template (Figure 6.4).

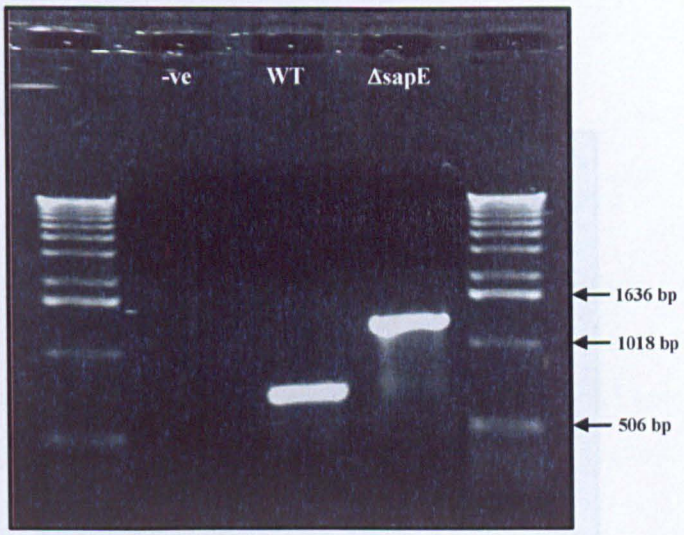

Figure 6.4 Confirmation of *Mc. capsulatus* Δ*sapE* genotype by PCR. Agarose (1% w/v) gel containing PCR products amplified from DNA extracted from *Mc. capsulatus* wild-type strain and *Mc. capsulatus* Δ*sapE* strain, which yielded 697 bp and 1247 bp PCR products, respectively. The size difference of the PCR products confirmed the genotype of the *Mc. capsulatus* Δ*sapE* mutant strain.

In addition to the PCR confirmation of the genotype of the Δ*sapE* mutant strain, the removal and thus the knock-out of the internal DNA portion of the *sapE* gene in the mutant strain was also confirmed by Southern hybridisation. PCR primers sapE-Out-F (5'-GTA GAC CGC ACC AAC GAT CC-3') and sapE-Out-R (5'-CGA ATG GCA CGG TGA ACA GG-3') were designed to amplify a 229 bp DNA fragment from within the knock-out region. The PCR product was then subsequently used as a probe for probing a Southern blot containing DNA extracted from *Mc. capsulatus* wild-type strain and Δ*sapE* strain as described in Section 2.7.4 (Figure 6.5).

The Southern blot clearly confirmed the removal of the internal DNA portion of *sapE* gene in the Δ*sapE* mutant strain, since no hybridisation signals were observed with DNA extracted from the mutant strain following Southern hybridisation with the knock-out region.

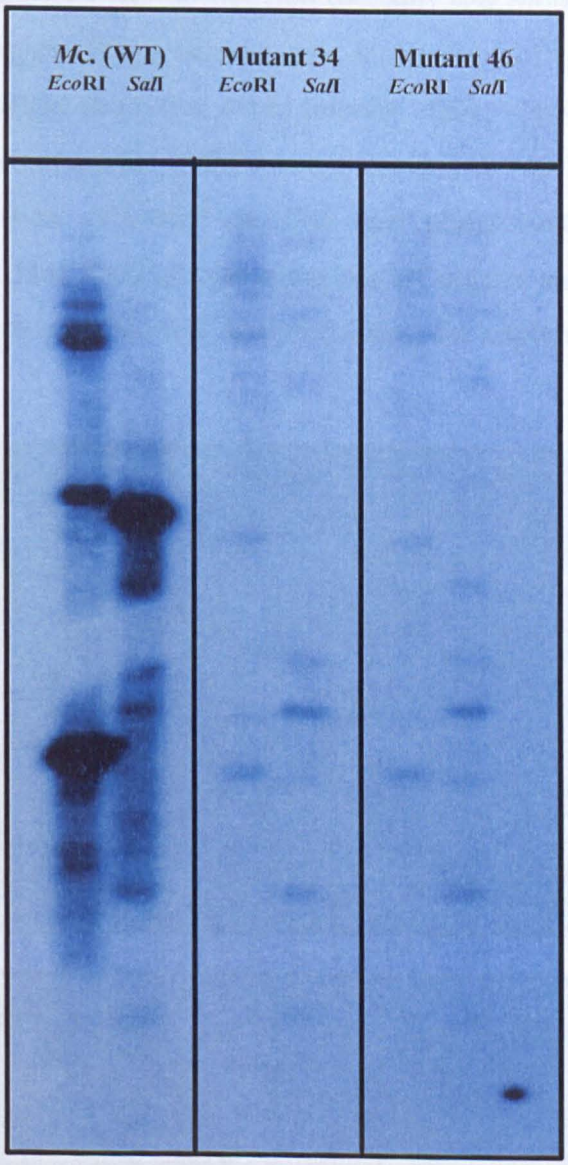

Figure 6.5 Confirmation of *Mc. capsulatus* Δ*sapE* genotype by Southern hybridisation. Southern blot containing *Eco*RI and *Sal*I DNA digests of *Mc. capsulatus* wild-type and Δ*sapE* strain hybridized with the knock-out region of *sapE*. Note: DNA was extracted from two independent Δ*sapE* mutant strains (Transconjugant 34 and 46), which were analysed for the removal of the knock-out region.

6.2.4 Phenotypic characterisation of SapE mutant

One of the hypotheses for the function of SapE and MopE was that they are involved in copper transport. If this was the case, inactivation of either SapE or MopE would give rise to a mutant strain that could tolerate higher copper concentration for methane oxidation when using the sMMO enzyme. In order to test this hypothesis, Δ*sapE* mutant strains were cultivated on NMS agar plates containing high (1 μM $CuSO_4$) and low (no added $CuSO_4$) concentrations of copper and the ability of the mutant strains to express sMMO were analysed using the naphthalene assay (Figure 6.6).

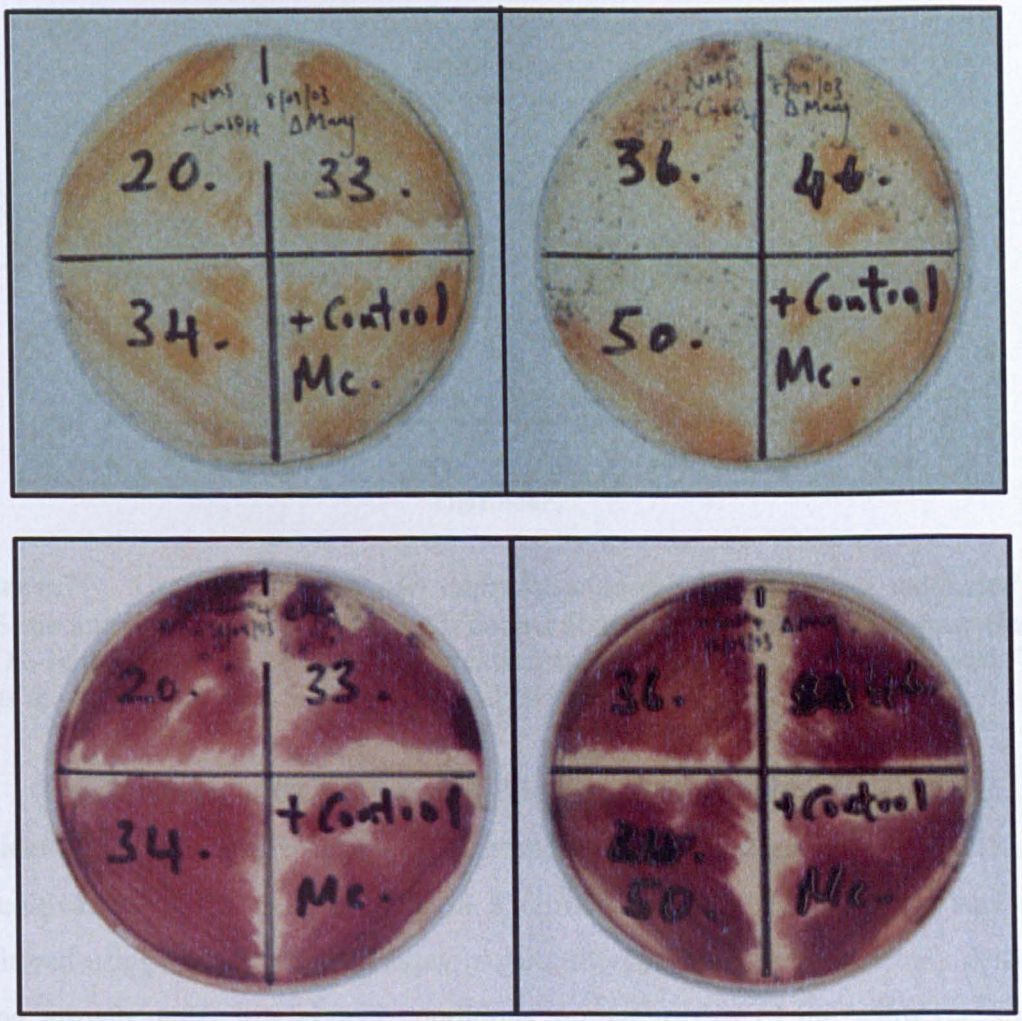

Figure 6.6 sMMO activity of *Mc. capsulatus* wild-type (positive control) and Δ*sapE* mutant strain grown on NMS agar plates containing high (top two plates) and low (bottom two plates) concentrations of copper. sMMO activity was tested using the naphthalene assay. The appearance of purple colonies indicates sMMO activity and thus sMMO expression.

The naphthalene assay confirmed the ability of the mutant strains to express sMMO under low concentrations of copper. Furthermore, the mutant strains defective in *sapE* did not have an increased capability to tolerate copper for sMMO expression, since on NMS agar plates containing 1 μM $CuSO_4$, no sMMO expression could be detected with the naphthalene assay. In addition to these data, the mutant strain was cultivated in a 5 L fermentor in batch cultures to investigate the effect of the disruption of SapE on growth. Initially the mutant strain was cultivated in NMS medium containing 1 μM $CuSO_4$ and then on no added copper medium in the second batch (Figure 6.7).

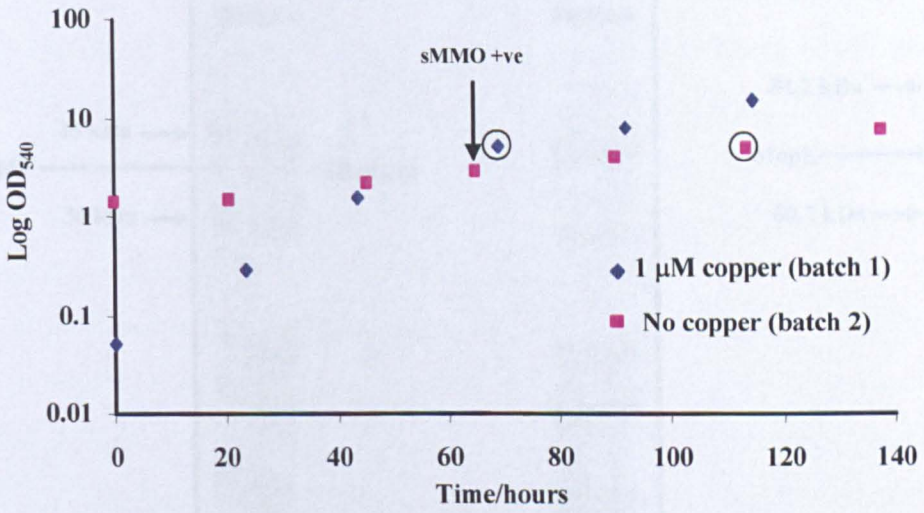

Figure 6.7 Growth curve of *Mc. capsulatus* Δ*sapE* mutant strain cultivated on NMS medium containing 1 μM $CuSO_4$ copper (batch 1) and no added copper (batch 2). The initial detection of sMMO expression by naphthalene assay is indicated. The points at which cells were removed for analysis are indicated by circles.

The disruption of the *sapE* gene in *Mc. capsulatus* Δ*sapE* mutant strain did not considerably affect the growth under high and low concentrations of copper. During the cultivation of the mutant strain in a fermentor, the sMMO activity was also monitored using the naphthalene assay. Again, the sMMO activity was only detected during growth under low copper conditions similar to that of the wild-type strain, which complemented the data presented in Figure 6.6. Although the disruption of the *sapE* gene did not affect the expression of MMO or the copper tolerance for sMMO expression to an appreciable level, the affect on the expression of MopE and the proteolysis of MopE to MopE* was nevertheless further investigated.

The pMMO and sMMO expressing cells of *Mc. capsulatus* Δ*sapE* mutant strain, obtained from fermentor cultures (Figure 6.7), were spun down and the respective supernatants were concentrated using Microcon centrifugal filter columns (Millipore). The concentrated supernatants were analysed for the secreted protein MopE* by SDS-PAGE (Figure 6.8A). In addition, whole cell lysates were analysed for the expression of MopE by Western hybridization using an anti-MopE serum, which was done by Anne Fjellbirkeland (University of Bergen) (Figure 6.8B).

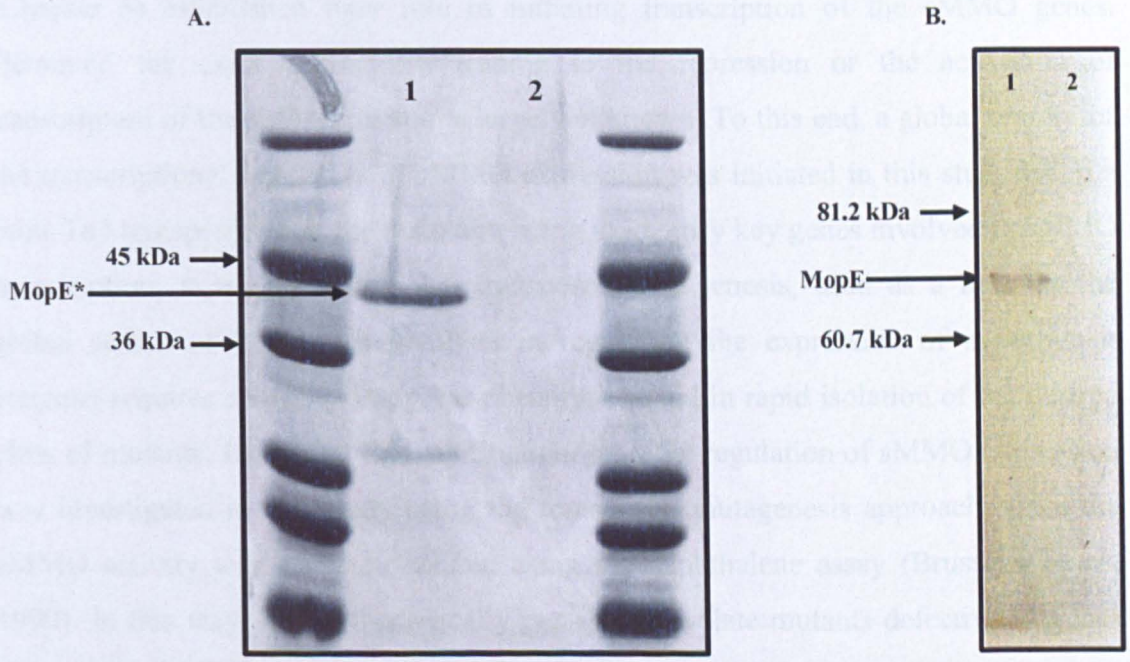

Figure 6.8 Analysis of the expression of MopE and the secretion of MopE* in *Mc. capsulatus* Δ*sapE* mutant strain. A. SDS-PAGE analysis of the secreted protein MopE* in supernatant obtained from cells cultivated on low (lane 1) and high (lane 2) concentrations of copper. B. Analysis of MopE expression by Western blotting using anti-mopE serum in whole cell lysates prepared from cells cultivated on low (lane 1) and high (lane 2) concentrations of copper. In each case the MopE* (43 kDa) and MopE (66 kDa) are indicated.

The detection of the expression of MopE and MopE* in *Mc. capsulatus* Δ*sapE* mutant strain indicated that SapE was not involved in the proteolysis of MopE. This was contrary to the initial hypothesis of the function of SapE as discussed above and the data presented in Figure 6.8 were similar to those data obtained using the wild-type strain (Fjellbirkeland *et al.*, 2001; Karlsen *et al.*, 2003). Despite this thorough analysis of the Δ*sapE* mutant strain, no obvious altered phenotype was observed.

6.3 Transposon mutagenesis using mini-Tn*5*

6.3.1 Introduction

The data presented in Chapter 4 and Chapter 5 reaffirmed the unique 'copper-switch' regulating the transcription of the sMMO operon and thus sMMO expression (Nielsen *et al.*, 1996). The identification of *mmoR* and *mmoG* and the subsequent mutational (Csaki *et al.*, 2003; Stafford *et al.*, 2003) and transcriptional analysis (Chapter 5) established their role in initiating transcription of the sMMO genes. However, the exact mechanism leading to the repression or the activation of transcription of the sMMO operon is largely unknown. To this end, a global search for the transcriptional regulation of sMMO expression was initiated in this study using a mini-Tn*5* transposon with the main aim being to identify key genes involved in sMMO transcription. It is noteworthy that transposon mutagenesis, used as a tool for the global search of key genes involved in regulating the expression of a particular enzyme, requires an easily assayable phenotype to aid in rapid isolation of the desired class of mutants. In light of this information, only the regulation of sMMO expression was investigated in this study using the transposon mutagenesis approach, since the sMMO activity was easily detectable using the naphthalene assay (Brusseau *et al.*, 1990). In this way, it was theoretically possible to isolate mutants defective in genes that are involved at any stage of sMMO regulation.

In this study, a mini-Tn*5* derivative designated as pBSL202 was used to carry out transposon mutagenesis since it has been widely used for mutagenesis by insertional inactivation and also for insertion of cloned DNA into chromosome (Alexeyev *et al.*, 1995) (Figure 6.9). The pBSL202 transposon vector was ideal for this study since it was based on a conditional origin of replication from plasmid R6K and thus was unable to replicate in methanotrophs. In addition, it had an origin of transfer from the broad host range plasmid RP4 allowing the transposon vector to be conjugally transferred to a wide range of Gram-negative bacteria. The important feature of pBSL202 was that it contained a gentamicin resistance gene within the transposable element, which was flanked by the 19 bp inverted repeat sequence that is important for transposition (Alexeyev *et al.*, 1995) and the transposase gene lay outside the inverted repeat sequence. This feature of the transposon vector prevented secondary transpositions, deletions, and inversions (Delorenzo *et al.*, 1990) due to the

loss of the plasmid backbone carrying the transposase gene. Thus transposon mutants generated with pBSL202 were generally more stable and only one transposon was inserted per cell, making the downstream analysis less ambiguous.

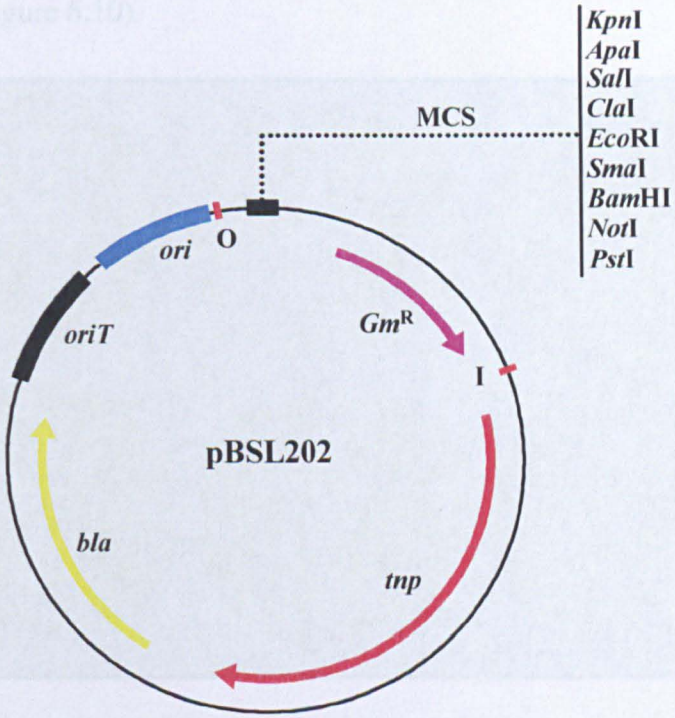

Figure 6.9 Physical map of mini-Tn*5* transposon vector, pBSL202 used for insertion mutagenesis described by Alexeyev *et al.*, (1995). Note: The O and I regions indicate the inverted repeat sequence of Tn*5* and it constitutes the transposable region, which contains a gentamicin resistance gene.

6.3.2 Mutagenesis

The transposon mutagenesis experiment was carried out in collaboration with Robert Csaki (University of Szeged) where pBSL202 was conjugated into *Mc. capsulatus* using a similar method described in Section 2.5.1. Initially, 10,000 mutants were selected on replica NMS agar plates containing gentamicin (10 μg ml^{-1}) and screened for sMMO expression using the naphthalene assay. From this screen, 200 putative sMMO minus mutants were identified, of which only 22 survived. As part of this study, the 22 putative sMMO minus mutants, isolated by Robert Csaki were further characterised by locating the region of Tn*5* insertion within the chromosome of *Mc. capsulatus*.

6.3.3 Analysis of the putative sMMO minus Tn*5* mutants

The 22 putative sMMO minus Tn*5* mutants isolated by Robert Csaki were re-analysed, using the naphthalene assay, for their ability to oxidise methane using the sMMO enzyme (Figure 6.10).

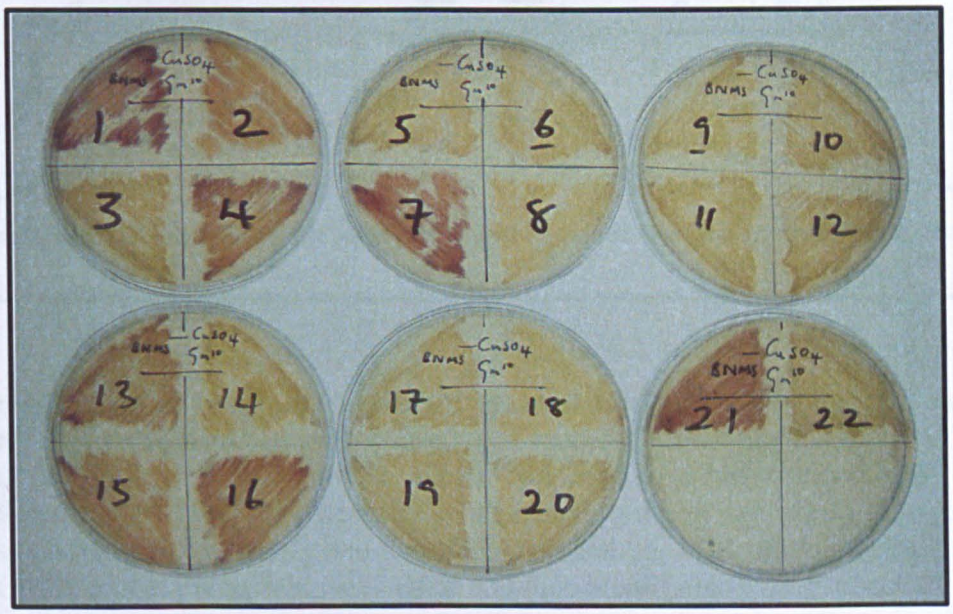

Figure 6.10 Analysis of the putative sMMO minus transposon mutants for sMMO expression using the naphthalene assay. Note: The mutants were cultivated on NMS agar plates containing gentamicin (10 μg ml^{-1}) and had no added copper. The purple colonies indicate sMMO expression.

The re-analysis of the transposon mutants indicated that some of the mutants initially identified as sMMO minus mutants (Δ1, 4, 7, 13, 15, 16, and 21) were in fact not sMMO minus since they were capable of oxidising naphthalene and thus capable of expressing sMMO. This indicated that the naphthalene assay was prone to yielding false negative results. The sensitivity of the naphthalene assay was indeed a limitation of this assay system. Nevertheless, most of the transposon mutants had an sMMO minus phenotype. In order to further characterise and identify the inactive gene responsible for exerting the sMMO minus phenotype, the Tn*5* transposon and the flanking genomic DNA was cloned using an inverse PCR method described in Section 2.6.1.

Genomic DNA was isolated from the transposon mutants and inverse PCR was carried out on circularised genomic DNA fragments using PCR primer Gm-F (5'-CCG

CGC TCA TCA ATC TCC TC-3') and Gm-R (5'-GGC TACGTC TCC GAA CTC AC-3'). The Gm-F and Gm-R primers were designed to anneal to the gentamicin resistance gene facing in the opposite orientation (Figure 6.11).

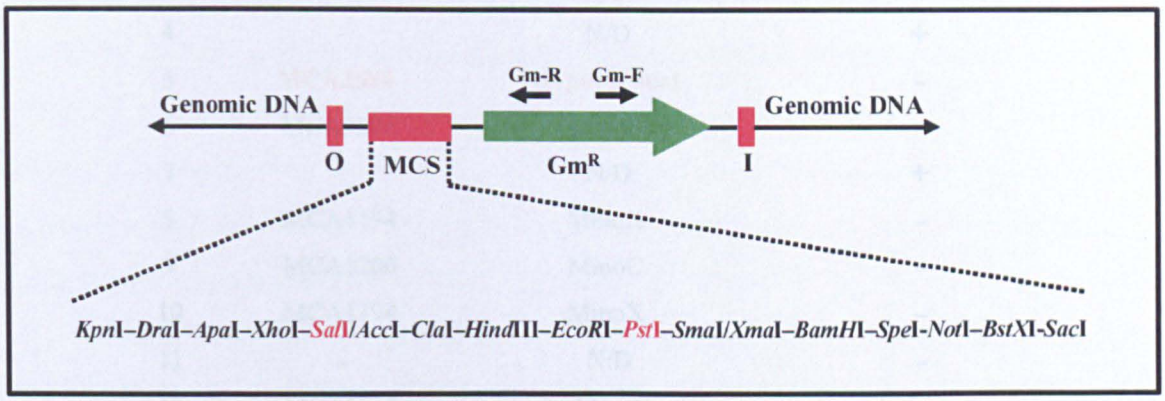

Figure 6.11 An example illustration of the Tn*5* integration into the chromosome. The gentamicin resistance gene was used as a marker to aid mutant isolation. Within the Tn*5* region a MCS was present, which consisted of a large selection of unique restriction sites. *Pst*I and *Sal*I were frequently used to digest *Mc. capsulatus* genomic DNA since they cut frequently and therefore the resulting PCR product using the Gm-F and Gm-R primers were relatively small and thus amplifiable.

The resulting PCR yielded products of various sizes, but products were not obtained for all of the mutants using the standard PCR conditions described in Section 2.6. However, following optimisation of the PCR conditions, PCR products were obtained for most of the mutants. The resulting PCR products were either sequenced directly using Gm-F or Gm-R primers or sequenced after cloning the PCR products into pCR2.1-TOPO vector as described in Section 2.4.6. The DNA sequence of the PCR products was analysed to locate the position of the Tn*5* insertion in the chromosome of *Mc. capsulatus*. Results are summarised in Table 6.2.

Mutant	Locus tag	Protein	sMMO Expression
1	MCA1204	MmoQ	+
2	MCA1394	Hypothetical protein	+
3	**MCA0866**	**Oxygen**[1]	-
4	-	N/D	+
5	**MCA2684**	**Hypothetical**	-
6	MCA1205	MmoR	-
7	-	N/D	+
8	MCA1194	MmoX	-
9	MCA1200	MmoC	-
10	MCA1194	MmoX	-
11	-	**N/D**	-
12	MCA1202	MmoG	-
13	MCA1894	Hypothetical	+
14	**MCA2684**	**Hypothetical**	-
15	MCA1394	Hypothetical	+
16	MCA1542	SdhB[2]	+
17	**MCA0866**	**Oxygen**[1]	-
18	-	**N/D**	-
19	MCA1194	MmoX	-
20	MCA1200	MmoC	-
21	MCA1469	σ^E Factor[3]	+
22	MCA1200	MmoC	-

[1]Oxygen-independent coproporphyrinogen III oxidase family protein

[2]Succinate dehydrogenase catalytic subunit

[2]Sigma-E factor regulatory protein

Table 6.2 Summary of the location of Tn*5* insertion within the chromosome of Δ1-Δ22 transposon mutants. The DNA sequence of the disrupted genes can be found by tracking the locus tags of the genes at NCBI under the accession number: NC_002977. The proteins encoded by the disrupted genes are also indicated. Interesting sMMO minus mutants (i.e. mutants resulting from insertional inactivation of genes other than sMMO structural genes or *mmoR* or *mmoG*) are highlighted in red. N/D, not done since no PCR products were obtained.

The characterisation of the transposon mutants and the identification of the location of Tn*5* insertion within the *Mc. capsulatus* chromosome gave further insights into their phenotype with respect to methane oxidation using the sMMO enzyme. The identification of *mmoQ* mutants (Δ1), which was capable of expressing sMMO indicated that *mmoQ* was not involved in sMMO expression. This result was in

accordance with data obtained by R. Csaki (University of Szeged) (Personal communication). Note: *mmoQ* encodes for a putative regulatory protein of a two-component sensor regulatory system. In addition, many of the other false sMMO negative mutants were found to be a result of insertional inactivation of non-essential genes for sMMO expression and encoded for hypothetical proteins whose functions were not known. However, the role of the genes inactivated in Δ16 and Δ21 were related to non-essential function for sMMO expression as they encoded for a putative flavoprotein (SdhB) subunit of succinate dehydrogenase[1] and a σ^E regulatory protein[2]; none of which are essential for sMMO expression.

Many of the sMMO minus mutants were a result of insertional inactivation of the structural genes (i.e. *mmoX* and *mmoC*) or insertional inactivation of the regulatory genes (i.e. *mmoR* and *mmoG*) and thus were not of interest for the purpose of this study. But they did confirm these genes as important regulators of sMMO expression. The identification of these sMMO minus mutants, however, reinforced the practicalities of this method as a system for the global search of genes involved in sMMO transcription and expression.

More interestingly, a number of additional sMMO minus mutants were identified, which were a result of insertional inactivation of genes other than the sMMO structural genes or the regulatory genes and are highlighted in red in Table 6.2. Unfortunately, the functions of the inactivated genes in most of these mutants were either not known or no PCR products were obtained. However, Δ3 and Δ17 were both a result of insertional inactivation of a gene encoding a putative oxygen-independent coproporphyrinogen III oxidase (CPOs) family protein, which resulted in a sMMO minus phenotype in both mutants. Oxygen-independent CPOs catalyse the oxidative decarboxylation of coproporphyrinogen-III to protoporphyrinogen-IX (Figure 6.12A), which is essential for the biosynthesis of the tetrapyrrole ring of heme.

In *E. coli,* the gene encoding oxygen-independent CPOs is termed *hemN*, which contains the conserved cysteine motif CXXXCXXCXC. These cysteine residues have been shown to be essential for oxygen-independent CPO in *E. coli* by site-directed

[1] Succinate dehydrogenase (SDH) is a membrane bound enzyme complex containing a flavour protein (Fp), a Iron-sulfur protein (Ip) and a cytochrome b_{558} (cyt*b*). SDH converts succinate to fumarate as part of the TCA cycle (Magnusson *et al.*, 1985) [2]The σ^E proteins have been identified in *E. coli* and *S. typhimurium* and are involved in the expression of genes determining resistance to heat and thus maintaining the integrity of the periplasmic and outer membrane components (Hiratsu *et al.*, 1995; Egler *et al.*, 2005).

mutagenesis and are thought to be essential for the formation of the iron-sulfur cluster required for enzyme function (Layer *et al.*, 2002). However, some bacteria carry a *hemN*-like gene termed *hemZ*, which contains only the first three cysteine residues (Layer *et al.*, 2002). In an alignment with other oxygen-independent CPOs, the conserved CXXXCXXC *hemZ* motif was also found in MCA0866 (Figure 6.12B).

Hemes are essential protein prosthetic groups for metabolism in all living organisms (Breckau *et al.*, 2003). Therefore, it can be postulated that the putative *hemZ* –like gene in *Mc. capsulatus* may encode for a heme protein needed for delivery of oxygen to sMMO, which is required for the initial oxidation of methane to methanol (Hanson & Hanson, 1996). Alternatively, it may be involved in the insertion of the essential metal iron centers, found within the α-subunit of the hydroxylase (Elango *et al.*, 1997). For the aims and purposes of this study, the role of MCA0866 was not further investigated.

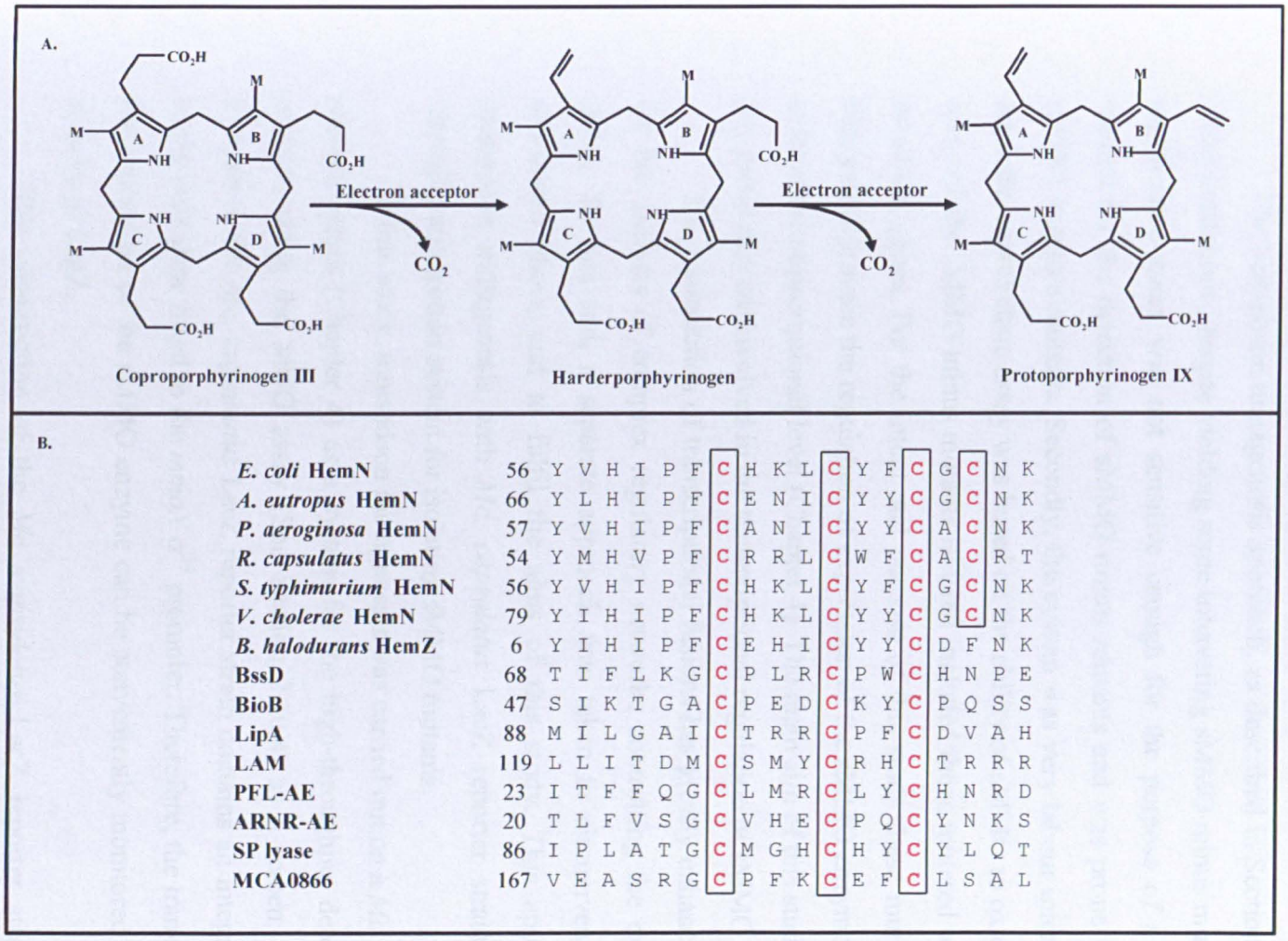

Figure 6.12 A. Oxidative carboxylation of coproporphyrinogen-III to protoporphyrinogen-IX by conversion of the propionate side chains of coproporphyrinogen-III ring A and B to vinyl groups. B. Amino acid alignments of HemN, HemZ and as well as other proteins containing the CXXXCXXC motif (i.e. *Thauera aromatica* benzylsuccinate synthase-activating enzyme (*BssD*), *E. coli* biotin synthase (*BioB*), *E. coli* lipoate synthase (*LipA*), *Clostridium subterminale* lysine 2,3 aminomutase (*LAM*), *E. coli* pyruvate formate-lyase activating enzyme (*PFL-AE*), *E. coli* anaerobic ribonucleotide reductase-activating enzyme (*ARNR-AE*), and *B. halodurans* spore photoproduct lyase (*SP lyase*). The amino acid sequences were taken from Layer *et al.*, (2002). The conserved residues are highlighted in red.

6.4 Transposon mutagenesis using a LacZ reporter strain

6.4.1 Introduction

The transposon mutagenesis approach, as described in Section 6.3, highlighted a few limitations, despite yielding some interesting sMMO minus mutants. Firstly, the naphthalene assay was not sensitive enough for the purpose of a high-throughput method for the detection of sMMO minus mutants and was prone to isolating false sMMO negative mutants. Secondly, the system was very labour intensive and thirdly, since the naphthalene assay was based on the ability of sMMO to oxidise naphthalene, many of the sMMO minus mutants isolated included those mutated within the sMMO structural genes. For the intent and purpose of this study these mutants were not of high priority, since the regulation of expression of the sMMO enzyme has been shown to be at the transcriptional level (Chapter 4). The main aim of this study was to identify key genes that are involved in the transcriptional regulation of sMMO enzyme.

The construction of transcriptional fusions has greatly enhanced the procedure for the analysis of complex regulatory networks controlling the expression of key genes. To this end, a separate approach was taken to circumvent the limitations mentioned above and to fulfil the aims of this study. This approach combined transposon mutagenesis with *Mc. capsulatus* LacZ reporter strain to aid a high-throughput detection system for isolating sMMO mutants.

In this study, transposon mutagenesis was carried out on a *Mc. capsulatus* LacZ reporter strain (Chapter 4) as a system for the high-throughput detection of sMMO mutants using the MUG assay (See Section 2.10.4) as a screen. As described in Chapter 4, the *Mc. capsulatus* LacZ reporter strain contains an integrated single copy of the *lacZ* gene fused to the *mmoX* σ^{54} promoter. Therefore, the transcription and thus the expression of the sMMO enzyme can be conveniently monitored by following the activity of LacZ.

The construction of the *Mc. capsulatus* LacZ reporter strain involved the integration of the suicide promoter probe vector, pMHA034 into the chromosome, which was selected on gentamicin. Therefore the transposon vector pBSL202, as used in Section 6.3, was not suitable for this part of this study since transposition of Tn*5* was also selected on gentamicin thus preventing the direct selection of transposon mutants. This problem was overcome by using an alternative transposon vector,

pAG408 (Suarez *et al.*, 1997) which had similar features in common with pBSL202 (Figure 6.9 and Figure 6.13). In addition, pAG408 was suitable for the selection on kanamycin and thus allowed a counter selection against the reporter strain for the direct selection of transposon mutants.

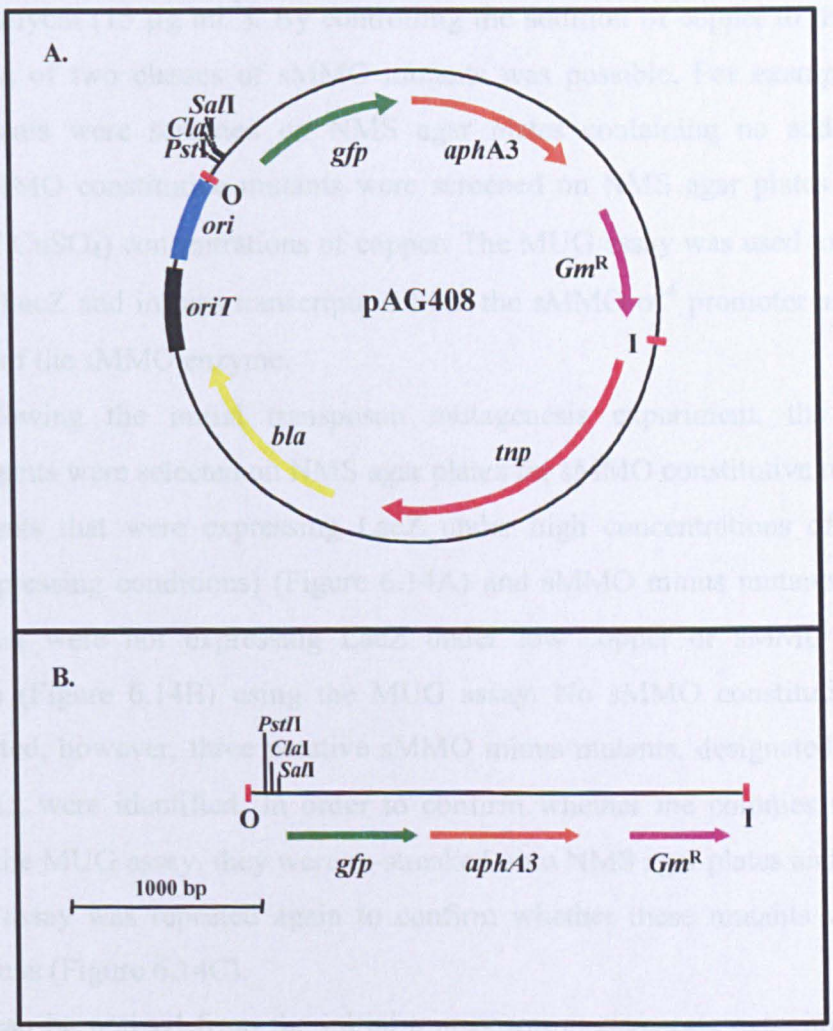

Figure 6.13 A. The genetic map of the promoter probe *gfp*-based mini-transposon vector, pAG408 (Suarez *et al.*, 1997). The *ori* was based on a conditional origin of replication from plasmid R6K and the *oriT* was from the broad host range plasmid RP4. B. The transposable element of pAG408, which contains *gfp*, *aphA3* conferring kanamycin resistance and Gm^R conferring gentamicin resistance. Note: Only those restriction sites which are unique within the mobile genetic element and in addition cut *Mc. capsulatus* genomic frequently are shown.

6.4.2 Mutagenesis, screening and isolation of sMMO mutants

The transposon mutagenesis experiments were performed by conjugation using 30 ml exponentially growing cultures (OD_{540} 0.2-0.6) of *Mc. capsulatus* LacZ reporter strain and 5 ml of overnight cultures of *E. coli* S17.1 λ*pir* strain containing pAG408 using the method described in Section 2.5.1. The resulting transconjugants or transposon mutants were selected on NMS agar plates containing gentamicin (5 μg ml^{-1}) and kanamycin (15 μg ml^{-1}). By controlling the addition of copper to the medium, the isolation of two classes of sMMO mutants was possible. For example, sMMO minus mutants were screened on NMS agar plates containing no added copper, whereas sMMO constitutive mutants were screened on NMS agar plates containing high (5 μM $CuSO_4$) concentrations of copper. The MUG assay was used to follow the activity of LacZ and in turn transcription from the sMMO σ^{54} promoter and thus the expression of the sMMO enzyme.

Following the initial transposon mutagenesis experiment, the respective transconjugants were selected on NMS agar plates for sMMO constitutive mutants (i.e. those mutants that were expressing LacZ under high concentrations of copper or pMMO expressing conditions) (Figure 6.14A) and sMMO minus mutants (i.e. those mutants that were not expressing LacZ under low copper or sMMO expressing conditions) (Figure 6.14B) using the MUG assay. No sMMO constitutive mutants were detected, however, three putative sMMO minus mutants, designated as mutants A, B and C, were identified. In order to confirm whether the colonies were viable following the MUG assay, they were re-streaked onto NMS agar plates and in addition the MUG assay was repeated again to confirm whether these mutants were indeed sMMO minus (Figure 6.14C).

It can be noticed from the initial transposon mutagenesis experiment that the frequency of transposition (i.e. the number of transposon mutants per ml of viable *Mc. capsulatus* cells) was very low (2.05×10^{-8}). In order to optimise and thus improve the frequency of transposition, a number of conjugations were carried out with cultures of *Mc. capsulatus* LacZ reporter strain taken from early, mid and late exponential growth phase. The highest frequency of transposition was yielded when conjugations were carried out with *Mc. capsulatus* cells taken from early (OD_{540} 0.2) exponential growth phase, which had a frequency of transposition of 2.72×10^{-7} (Figure 6.14D). This was

an order of magnitude higher than the initial experiment where *Mc. capsulatus* cells were taken from late (OD_{540} 0.6) exponential growth phase.

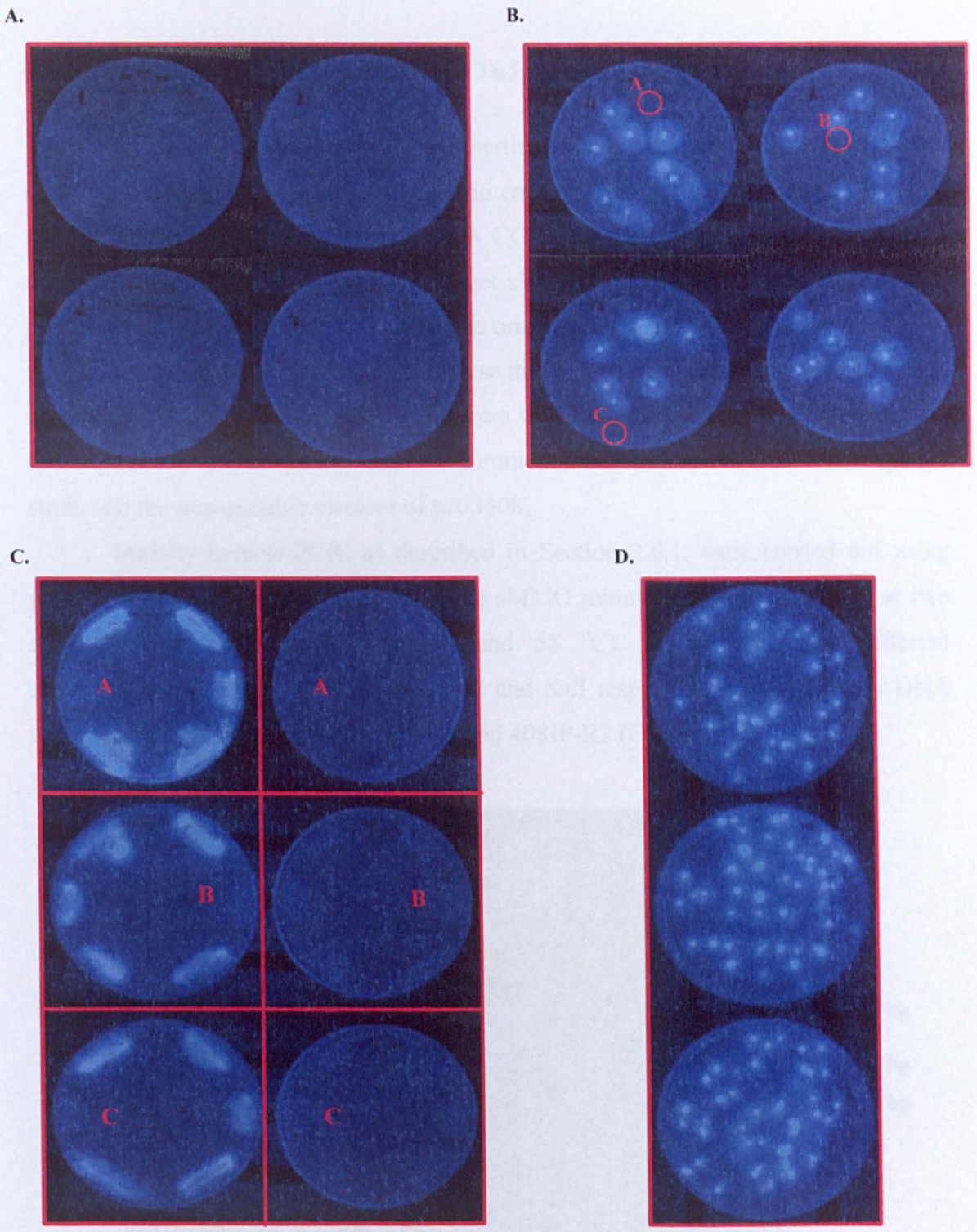

Figure 6.14 Screening transconjugants for sMMO mutants using the MUG assay. Transposon mutants selected on NMS agar plates containing high (5 μg ml^{-1}) concentrations of copper for screening sMMO constitutive mutants (A), no added copper plates for screening sMMO minus mutants (B). The putative sMMO minus mutants identified are highlighted by red circles (C). The viability and the sMMO minus phenotypes were re-confirmed by streaking colonies onto NMS agar plates containing high (plates on the left hand side) and no added (plates on the right hand side) copper following MUG assay. Note: a number of sMMO positive mutants capable of expressing LacZ were included on each mutant plate as positive control. An improved frequency of transposition was achieved using *Mc. capsulatus* LacZ cells taken from early exponential growth phase (D).

6.4.3 Identification and localisation of Tn*5* insertion

To identify the region of Tn*5* insertion within the chromosome of the *Mc. capsulatus* LacZ reporter strain, new primers, 408IP-F (5'-GGT CGA TCA GGG AGG ATG TC-3') and 408IP-R2 (5'-TCA CCT TCA CCC TCT CCA CTG-3') were designed. These primers annealed to target sites within the transposable element of pAG408 (Figure 6.13B) and in the opposite orientation. Note: It was not suitable to use the Gm-F and Gm-R primers used to analyse the Tn*5* location within the sMMO minus mutants in Section 6.3.3, since these primers would yield multiple PCR products due to the presence of Gm^R gene within the chromosome of *Mc. capsulatus* LacZ reporter strain and the transposable element of pAG408.

Initially inverse-PCR, as described in Section 2.6.1, were carried out using genomic DNA extracted from the putative sMMO minus mutants A, B and C at two different annealing temperatures (56 and 58 °C). In addition, two different recircularised genomic DNA digests (*Pst*I and *Sal*I respectively) were used as DNA template for PCR using primers 408IP-F and 408IP-R2 (Figure 6.15).

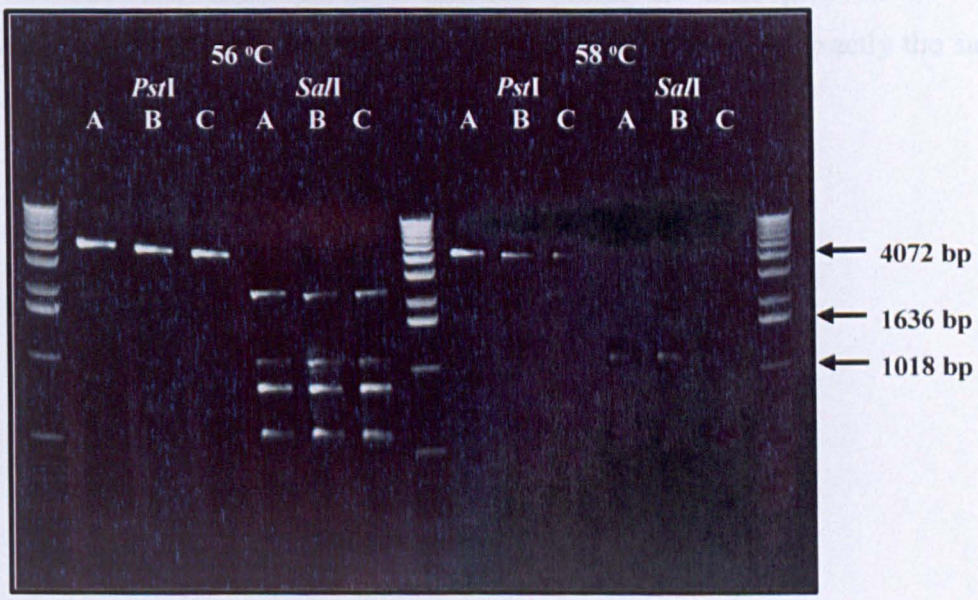

Figure 6.15 Analysis of Tn*5* insertion in the chromosome of *Mc. capsulatus* LacZ by inverse PCR. Agarose gel electrophoresis of inverse PCR products using DNA extracted from the putative sMMO minus mutants A, B and C. PCR reactions carried out at 56 °C and 58 °C and with *Pst*I and *Sal*I digested genomic DNA recircularised.

The PCR products obtained from both PCR reactions using two different digests of genomic DNA yielded identical sizes of DNA fragments. This was very surprising and therefore this was further investigated by Southern hybridisation using methods described in Section 2.7. A Southern blot was prepared, which consisted of genomic DNA fragments of the putative sMMO minus mutants digested with *Pst*I and *Sal*I respectively. The Southern blot was subsequently probed with a 1670 bp fragment of the mobile genetic element of the pAG408 transposon vector, which was labelled with ^{32}P by random labelling (Section 2.7.4) (Figure 6.16).

The Southern blot confirmed the inverse PCR data (Figure 6.15), which suggested that the Tn*5* had hot-spotted in one particular region of the chromosome. In addition, it confirmed that only a single copy of Tn*5* integrated into the chromosome. The exact location of the Tn*5* insertion within the chromosome of the putative sMMO minus mutants was confirmed. This was done by pooling the respective PCR products yielded from both PCR reactions using the *Pst*I digest recircularised, which were sequenced using the internal primers, Gm-F and Gm-R described above, prior to the purification of the PCR products using the methods described in Section 2.4.3.

The Tn*5* was found to have inserted within the *lacZ* gene in all three transposon mutants. In the case of mutant B and C, Tn*5* inserted in exactly the same position within the *lacZ* gene (Figure 6.17).

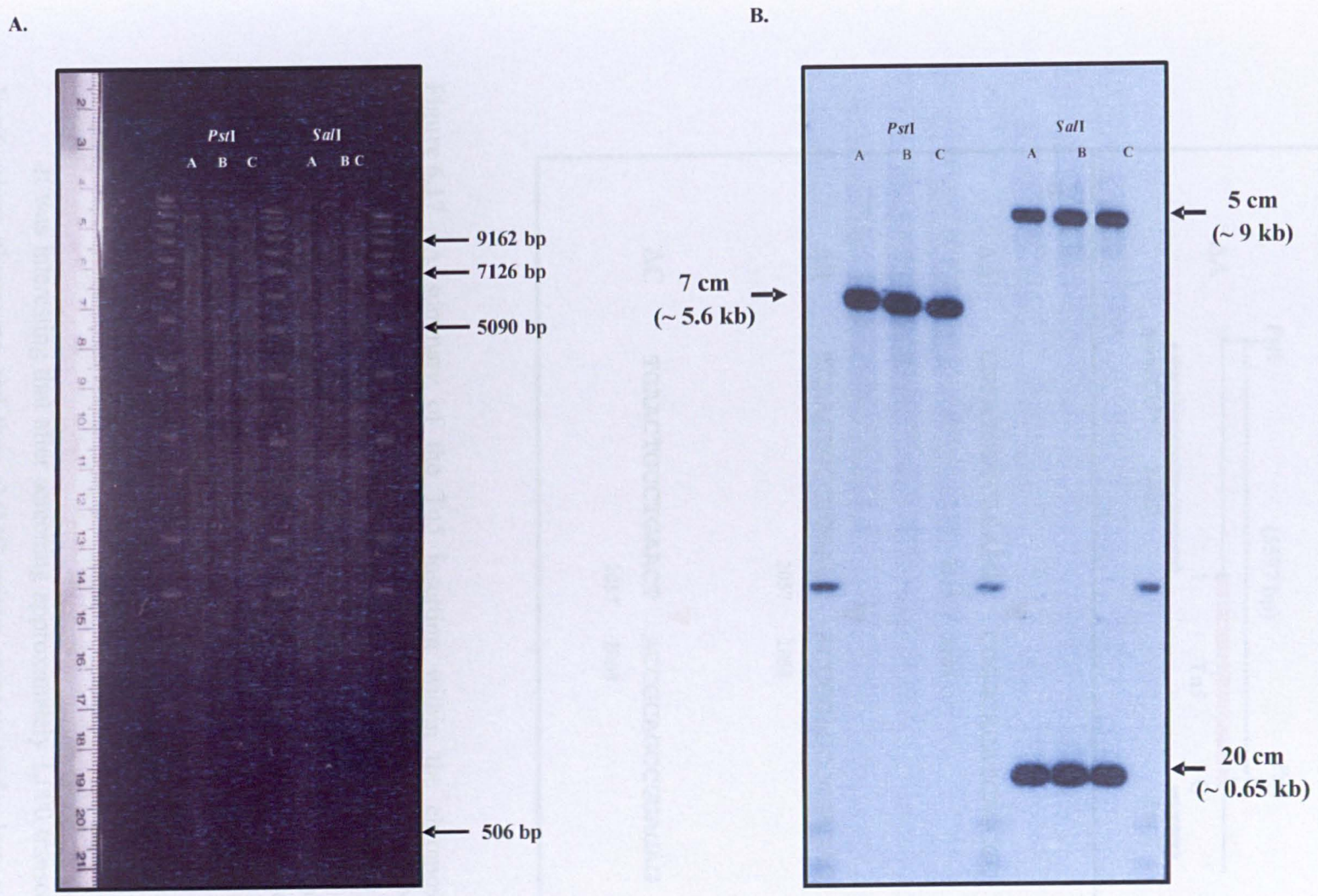

Figure 6.16 Analysis of Tn*5* insertion in the chromosome of the putative sMMO minus transposon mutants, A, B and C by Southern hybridisation. A. Agarose (0.9 % w/v) gel containing genomic DNA of *Mc. capsulatus* LacZ transposon mutants digested with *Pst*I and *Sal*I. C. The respective Southern blot hybridised with a 1670 bp *Kpn*I fragment of the mobile genetic element of pAG408 transposon vector. The approximate sizes of the DNA fragments hybridised with the probe are shown.

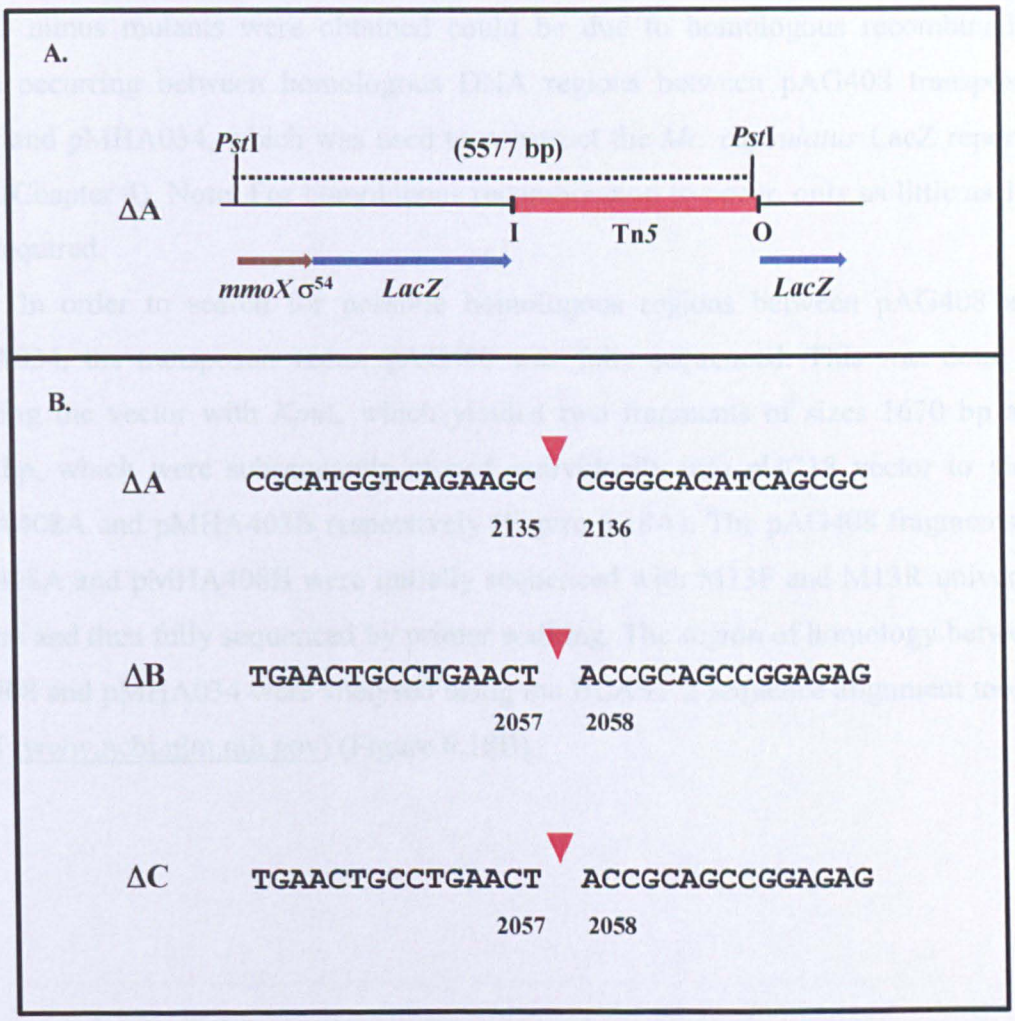

Figure 6.17 A summary of the Tn*5* insertion within the chromosome of the transposon mutant A, B and C. A. Schematic representation of the DNA region in mutant A following Tn*5* insertion within the *lacZ* gene. The dotted line between the *Pst*I sites indicates the approximate 5.6 kb DNA fragment detected by Southern hybridisation. B. Summary of Tn*5* insertion within the *lacZ* gene and the exact location of insertion are indicated by the nucleotide number with respect to the ATG of *lacZ* gene.

6.4.4 Sequence analysis of pAG408 and pMHA034

It was interesting that after screening approximately 1,000 transconjugants for LacZ minus phenotype and thus sMMO minus mutants under low copper growth conditions, only three putative sMMO minus mutants were identified, which turned out to be a result of insertional inactivation of the *lacZ* gene. One explanation, (apart from the possibility that not enough transconjugants were screened) why no genuine

sMMO minus mutants were obtained could be due to homologous recombination events occurring between homologous DNA regions between pAG408 transposon vector and pMHA034, which was used to construct the *Mc. capsulatus* LacZ reporter strain (Chapter 4). Note: For homologous recombination to occur, only as little as 100 bp is required.

In order to search for possible homologous regions between pAG408 and pMHA034, the transposon vector pAG408 was fully sequenced. This was done by digesting the vector with *Kpn*I, which yielded two fragments of sizes 1670 bp and 4498 bp, which were subsequently cloned individually into pUC18 vector to yield pMHA408A and pMHA408B respectively (Figure 6.18A). The pAG408 fragments in pMH408A and pMHA408B were initially sequenced with M13F and M13R universal primers and then fully sequenced by primer walking. The region of homology between pAG408 and pMHA034 were analysed using the BLAST 2 sequence alignment tool at NCBI (www.ncbi.nlm.nih.gov) (Figure 6.18B).

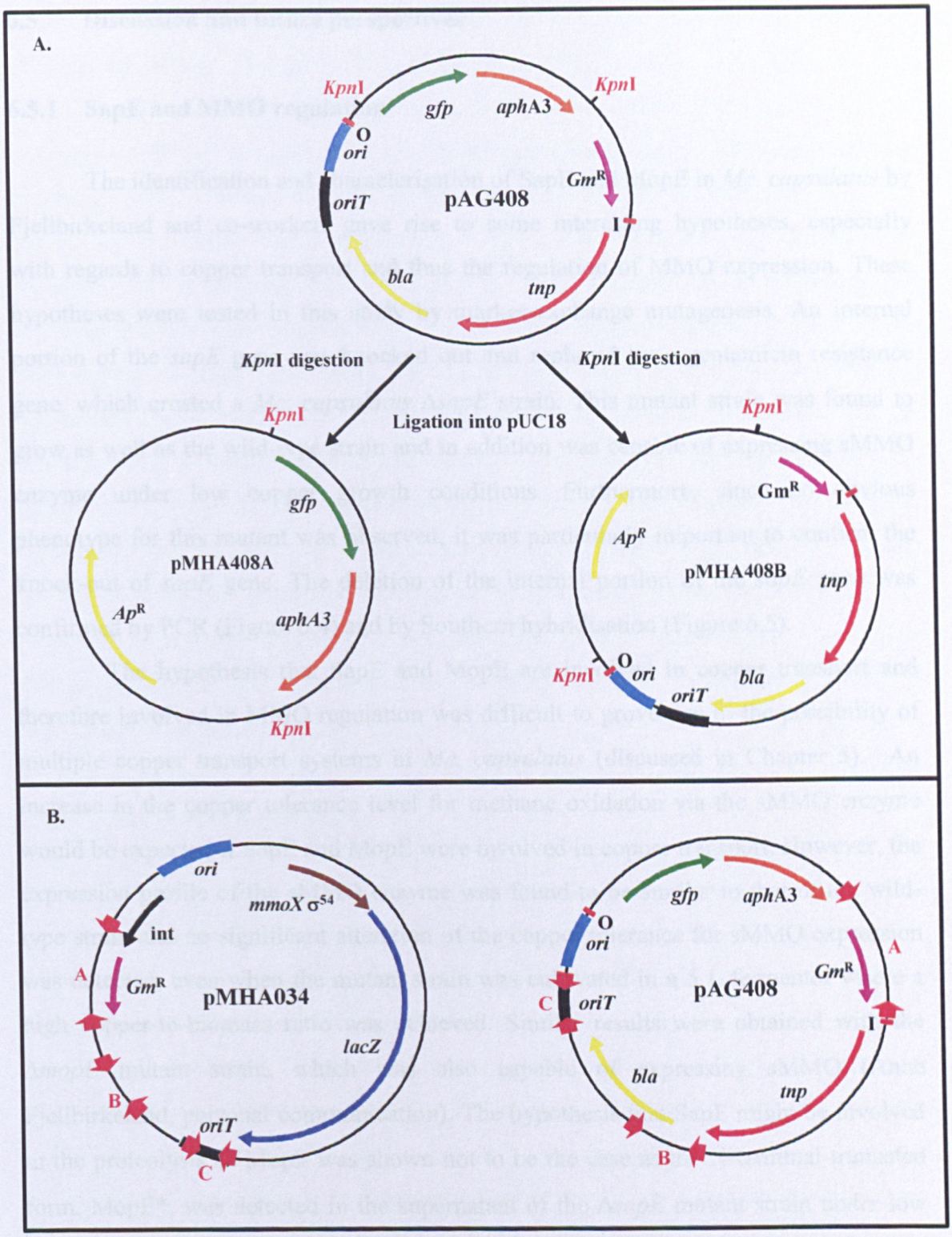

Figure 6.18 Identification of homologous regions between pAG408 and pMHA034. A. Construction of pMHA408A and pMHA408B, which were used to sequence pAG408. B. Genetic map of pAG408 and pMHA034 and the homologous regions between the two vectors, designated as regions A, B, and C, are indicated by the red arrows. The lengths of the homologous regions are as follows: A = 785 bp, B = 411 bp, and C = 276 bp.

6.5 Discussion and future perspectives

6.5.1 SapE and MMO regulation

The identification and characterisation of SapE and MopE in *Mc. capsulatus* by Fjellbirkeland and co-workers gave rise to some interesting hypotheses, especially with regards to copper transport and thus the regulation of MMO expression. These hypotheses were tested in this study by marker-exchange mutagenesis. An internal portion of the *sapE* gene was knocked out and replaced by a gentamicin resistance gene, which created a *Mc. capsulatus* Δ*sapE* strain. This mutant strain was found to grow as well as the wild-type strain and in addition was capable of expressing sMMO enzyme under low copper growth conditions. Furthermore, since no obvious phenotype for this mutant was observed, it was particularly important to confirm the knock-out of *sapE* gene. The deletion of the internal portion of the *sapE* gene was confirmed by PCR (Figure 6.4) and by Southern hybridisation (Figure 6.5).

The hypothesis that SapE and MopE are involved in copper transport and therefore involved in MMO regulation was difficult to prove due to the possibility of multiple copper transport systems in *Mc. capsulatus* (discussed in Chapter 5). An increase in the copper tolerance level for methane oxidation via the sMMO enzyme would be expected if SapE and MopE were involved in copper transport. However, the expression profile of the sMMO enzyme was found to be similar to that of the wild-type strain and no significant alteration of the copper tolerance for sMMO expression was detected, even when the mutant strain was cultivated in a 5 L fermentor where a high copper-to-biomass ratio was achieved. Similar results were obtained with the Δ*mopE* mutant strain, which was also capable of expressing sMMO (Anne Fjellbirkeland, personal communication). The hypothesis that SapE might be involved in the proteolysis of MopE was shown not to be the case as the N-terminal truncated form, MopE*, was detected in the supernatant of the Δ*sapE* mutant strain under low copper growth conditions. The secretion profile of MopE* in the mutant strain was similar to that of the wild-type strain (Karlsen *et al.*, 2003). In addition, the detection of MopE using anti-MopE serum in Δ*sapE* mutant suggested that SapE was also not involved in the expression of MopE. It is noteworthy that marker-exchange mutagenesis of the *sapE* gene did not create a polar affect on the transcription of *mopE*

nor did the in-frame introduction of a *Hind*III site within the *mopE* gene (Figure 6.2) affect its expression or the proteolysis of MopE.

In light of this information, it can be concluded that SapE is not involved in the proteolysis of MopE and also it does not play a significant role in MMO regulation. As of yet, the exact function of SapE remains to be elucidated. However, the effect of copper on the intracellular morphology with the increase in the formation of intracytoplasmic membranes in the presence of copper has been well established (Prior & Dalton, 1985). This coupled with the subcellular location and the expression of SapE under low copper conditions, leads to further speculation that SapE may serve a non-essential role under these physiological conditions, which is associated with the cell membrane. Furthermore, in a recent study, the amino acid sequence of SapE was shown to share significant sequence similarity to the bacterial di-heme cytochrome *c* peroxidase (BCCP) family of proteins (Karlsen *et al.*, 2005). In general, BCCPs are associated with the periplasm and serve in a protective role by reducing the peroxidases generated in oxidative metabolism (Goodhew *et al.*, 1990). This gave further support for the hypothesis that SapE is involved in a process which is associated with the membrane.

6.5.2 Transposon mutagenesis

The development of a transposon mutagenesis protocol in *Mc. capsulatus* and the subsequent identification of sMMO minus mutants using the naphthalene assay demonstrated the powerful nature of the transposon mutagenesis system for the global search for genes involved in MMO regulation. The naphthalene assay was useful as a screen since it did not discriminate between sMMO minus mutants resulting from insertional inactivation of genes involved sMMO transcription (i.e. *mmoR* and *mmoG*), sMMO structural genes (i.e. *mmoX* and *mmoC*) or genes involved in post-translational modification. The inability of the naphthalene assay to selectively discriminate between the different levels of sMMO minus mutants, coupled with the random mutagenesis using mini-Tn*5* transposon, made it theoretically possible to identify all the downstream genes required for the active expression of sMMO. However, the focus of this experiment was to selectively isolate mutants at the transcriptional level, therefore transposon mutagenesis coupled with the naphthalene assay added unnecessary complexity to this system. For example, the sMMO operon is ~5.5 kb and

therefore this was a large target for Tn*5* for inactivating sMMO expression. As it can be seen from Table 6.2, many of the genuine sMMO minus mutants were a result of insertional inactivation of sMMO structural genes. Therefore, in order to identify sMMO minus mutants resulting from insertional inactivation of genes involved in transcription of the sMMO operon, many sMMO minus mutants were required to be further analysed to locate the Tn*5* insertion within the chromosome. The naphthalene assay used as a screen for the global search for genes involved in the 'copper-switch' for the transcription of the sMMO operon added further to the labour intensiveness of this system. In addition, the complexity of the multicomponent sMMO enzyme that requires a fully assembled and maturated enzyme complex meant that the sensitivity of the naphthalene assay, which is dependent on the presence of active sMMO was low. The result of the low sensitivity of the naphthalene assay can be seen in Table 6.2 where a number of false sMMO minus mutants were isolated following the initial screen. Nevertheless, a number of interesting sMMO minus mutants other than the sMMO structural genes or sMMO regulatory genes were isolated. Two of these sMMO minus mutants (Δ3 and Δ17) were a result of insertional inactivation of MCA0866, which was annotated in the genome as a putative oxygen-independent coproporphyrinogen III oxidase (CPO) family protein (Ward *et al.*, 2004). In an alignment with other oxygen-independent CPO the conserved cysteine motif CXXXCXXC was identified and therefore it can be postulated that MCA0866 may play a role in the biosynthesis of a terminal oxygenase involved in the delivery and transport of dioxygen to the sMMO enzyme, which is required for the initial hydroxylation step for methane oxidation. Alternatively, MCA0866 could be involved in inserting the binuclear iron center within the α-subunit of the hydroxylase of the sMMO enzyme, which is thought to be the activation site for both dioxygen and methane (Elango *et al.*, 1997). Unfortunately, no putative function could be assigned to the other mutated gene (MCA2684) that resulted in an sMMO minus phenotype since it had no sequence similarity to other known proteins in the NCBI database and thus it was only assigned as a hypothetical protein. In addition, several attempts to obtain PCR products by inverse PCR to identify Tn*5* location in Δ11 and Δ18 were unsuccessful and therefore prevented further analysis of these sMMO minus mutants.

As discussed above, the basis for a successful transposon mutagenesis experiment very much depends on the assay system used for screening the desired

class of mutants. In an attempt to establish a high-throughput screening method for the rapid detection of sMMO minus mutants, transposon mutagenesis was carried out in conjunction with the *Mc. capsulatus* LacZ reporter strain, where the expression of LacZ was assayed using the MUG reagent to monitor transcription of the sMMO σ^{54} promoter. This approach allowed for the selective screening of sMMO minus mutants resulting only from insertional inactivation of genes involved in the transcriptional regulation of the sMMO operon and thus allowed a more focused approach for the global search of sMMO regulatory genes.

The identification of three LacZ minus mutants following the initial transposon mutagenesis demonstrated the high-throughput nature of this system as a screen for detecting sMMO mutants. Unfortunately, the subsequent analysis revealed the insertion of Tn*5* into a hot-spot within the *lacZ* gene. This kind of Tn*5* hot-spotting for a particular DNA site is not uncommon. The insertional specificity of Tn*5* has been studied in *E. coli* where it has been shown to have preferences for certain sites, however, Tn*5* was ranked amongst the Tn elements with the lowest insertional specificity (de Bruijn & Lupski, 1984). Berg *et al*, (1980) examined insertion specificity of Tn*5* in *E. coli lac* operon where they found less than 5 % of the transposon mutants to have Tn*5* inserted within the *lac* operon (Berg *et al.*, 1980). Similarly, strong hot-spots were identified in plasmid pBR322 (Berg & Berg, 1983). In light of this information, the Tn*5* hot-spot for the *lacZ* gene can be explained. In addition, the observation that only 3 LacZ::Tn*5* mutants out of the 1,000 were identified further suggests that the specificity of Tn*5* for *lacZ* was low and thus did not pose a major problem. Nevertheless, the slight affinity of Tn*5* for *lacZ* is indeed a limitation of this system, however, this limitation can be relatively easily overcome by pre-screening the LacZ minus mutants using the naphthalene assay, prior to analysing the mutants further for the location of Tn*5* insertion. Note: LacZ minus mutants will still be capable of expressing sMMO. This pre-screen will greatly reduce the number of mutants that need to be characterised and thus reduce the labour intensiveness of this system.

The DNA sequence comparison between the LacZ promoter probe vector pMHA034 and the transposon vector pAG408 revealed three homologous regions. This is potentially a problem, since it will increase the likelihood for recombination events to occur. The occurrence of recombination between the promoter probe vector and the transposon vector is one possible explanation why no LacZ minus mutants

were obtained that also had a sMMO minus phenotype. However a more likely explanation why no LacZ minus mutants were obtained could be down to the obligate nature of *M. capsulatus*. Although sMMO minus mutants are capable of growing under low copper conditions using the oxidation capacity of pMMO, the pMMO activity is known to decrease with decreasing copper concentration (Prior, 1985). As a result transposon mutants selected for LacZ minus and thus sMMO minus phenotype under low copper concentrations will generally be under a counter-selection pressure by those mutants not defective in sMMO expression.

6.5.3 Summary and future perspectives

The data presented in this study indicated that SapE was not involved in the proteolysis of MopE. In addition, there were no significant links between SapE and MMO regulation and thus for the intent and purpose of this study further experiments were abandoned. However, the features of MopE expression, particularly its ability to be specifically cleaved by an unknown mechanism and the C-terminal part of MopE to be secreted into the growth medium is being further exploited by Anne Fjellbirkeland (University of Bergen) as a vaccine delivery system for fish. The demonstration of MopE expression and the proteolysis of MopE* to be independent of SapE in this study will give further knowledge for the development of such vaccine delivery system.

In general, an experimental approach pursued for the global scale analysis of gene regulation needs to be considered carefully and will very much depend on the initial scientific problem or question. In summary, in this study a transposon mutagenesis approach was taken for the global search for genes involved in MMO regulation, where a protocol for random mutagenesis was developed. In addition, a number of interesting sMMO minus mutants have been identified. The power of transposon vectors as tools for genetic analysis was further extended in this study by combining it with a *Mc. capsulatus* LacZ reporter strain. This system allowed for high-throughput detection of sMMO minus mutants resulting from insertional inactivation of genes involved in the transcriptional regulation of the sMMO operon.

The transposon mutagenesis experiment based on the *Mc. capsulatus* LacZ reporter strain is set to yield some interesting data, which will give new insights into the molecular regulation of MMO expression after some minor modifications. To

avoid the possibility of recombination events to occur between homologous DNA regions present in pAG408 and pMHA034, pAG408 can be easily modified to remove these homologous regions (Figure 6.19). Furthermore, to simplify the downstream analysis of Tn*5* insertion within the chromosome, an additional conditional origin of replication can be engineered within the Tn*5* region. This will in effect create a plasposon vector, similar to those created by Dennis & Zylstra, (1998). The advantage of these vectors is that it will allow for the cloning of the Tn*5* region plus the flanking DNA region without carrying out inverse PCR. The digested chromosomal DNA can be simply recircularised and the respective circular DNA fragments can be used directly in transformation reactions in *E. coli*.

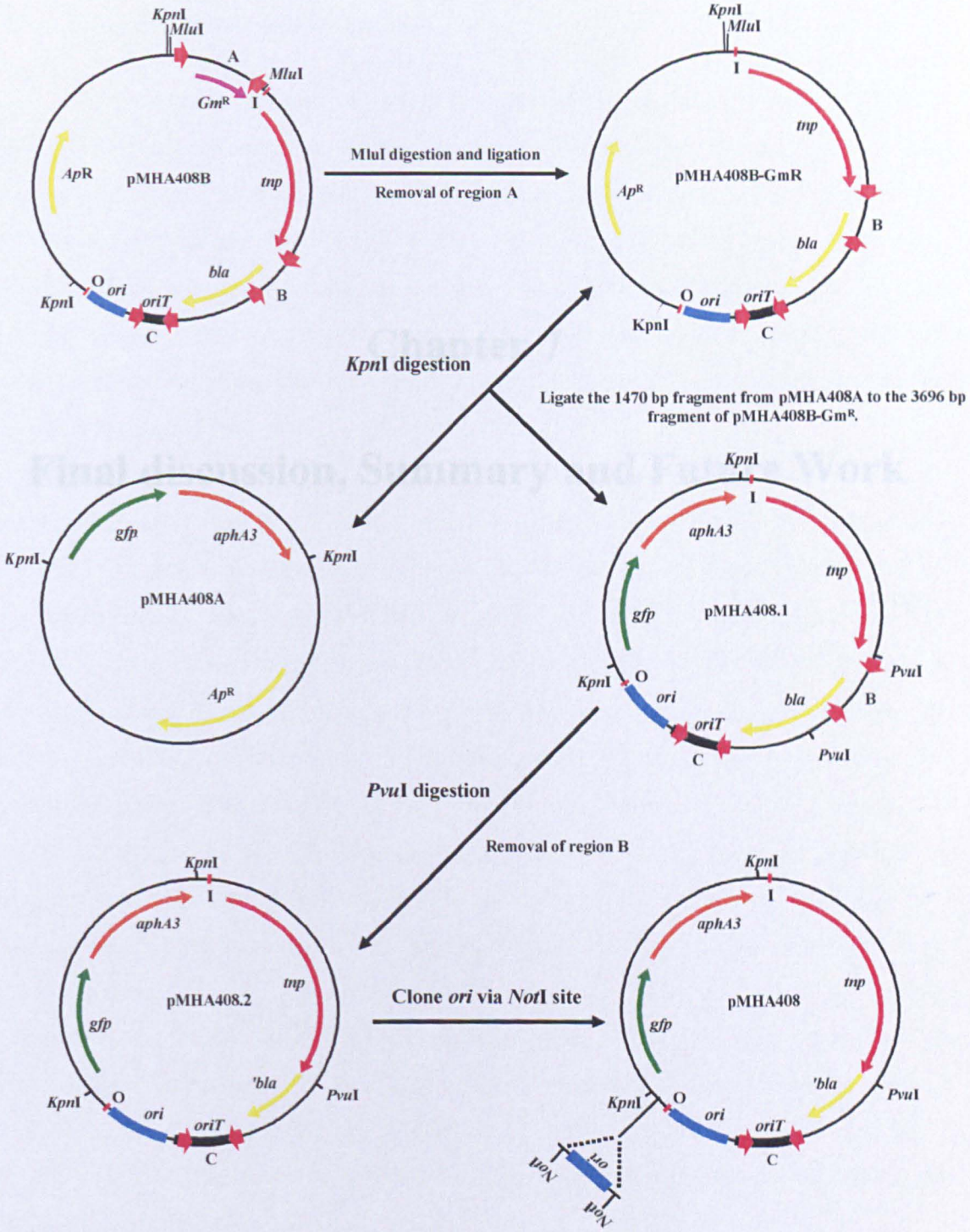

Figure 6.19 Schematic representation of the cloning strategy for the construction of a modified mini-transposon vector pMHA408.2 and a plasposon vector pMHA408. pMHA408A and pMHA408B were constructed earlier (See Figure 6.18).

Chapter 7

Final discussion, Summary and Future Work

7.1 Final discussion and summary

The molecular mechanism by which copper regulates the expression of pMMO and sMMO ('copper-switch') formed the basis of this project. At the start of this project, a great deal of molecular and biochemical information was known about the pMMO and sMMO enzymes, but only a limited number of useful genetic tools were available. This prevented detailed molecular studies from being carried out. To this end, the primary aims of this study were to further develop suitable genetic tools for methanotrophs and to use them to gain detailed insights into the molecular mechanism of MMO regulation by copper.

The initial identification of the sMMO regulatory genes, *mmoR* and *mmoG*, in *Mc. capsulatus* (Csaki *et al.*, 2003) and *Ms. trichosporium* (Stafford *et al.*, 2003) initiated the work presented in Chapter 3. Screening of type I and type II methanotrophs by Southern hybridisation using *mmoR* and *mmoG* as gene probes indicated their presence only in sMMO-expressing strains. The identification of duplicate *mmoX* genes in *Ms. sporium* led to the cloning, sequencing and the subsequent mutational analysis of the sMMO operon. Although both copies of *mmoX* gene were shown to be transcribed, only *mmoX*1 was essential for sMMO activity. In addition, construction of sMMO-minus mutant by marker-exchange mutagenesis gave insights into the role of the water soluble pigment in siderophore-mediated iron acquisition. *Ms. sporium* has been largely overlooked since its isolation more than three decades ago by Whittenbury and co-workers (Whittenbury *et al.*, 1970). However, mutagenesis studies and complementation of the sMMO-minus mutants by heterologous expression in this study demonstrated its amenability to genetic manipulation and opened up the possibility of using *Ms. sporium* as an alternative model organism for future exploration of the molecular mechanism regulating the expression of MMO.

The ability to monitor gene transcription under different physiological conditions represents an invaluable approach to understanding gene expression and regulation. In methanotrophs, the limited availability of suitable promoter probe vectors for carrying out transcriptional analysis of promoter elements initiated the work presented in Chapter 4, where a series of integrative suicide and broad host range promoter probe vectors were constructed. The usefulness of the integrative suicide vectors were examined by selecting *Mc. capsulatus* reporter strains carrying a

chromosomal fusion between *gfp*, *xylE*, km^R or *lacZ* gene with the sMMO σ^{54} promoter. The reporter gene activities were measured from cultures grown under high (pMMO expression conditions) and low (sMMO expressing conditions) concentrations of copper. The data obtained in all cases confirmed the copper repressible feature of the sMMO σ^{54} promoter and its strict regulation by copper at the transcriptional level. In addition, each reporter assay system was assessed for its suitability as a high-throughput screening method for detecting sMMO mutants. It was found that the β-galactosidase assay system based on MUG hydrolysis was the most effective method for measuring reporter gene activity in a high-throughput fashion, since it did not require cell lysis; it was quick, reliable and importantly very sensitive. The BHR promoter probe vectors were also shown to be an effective tool for transcriptional analysis as demonstrated in *M. silvestris* and *Mc. capsulatus*. In *M. silvestris*, a reporter strain carrying a plasmid containing a *gfp* gene fused to the sMMO σ^{54} promoter indicated the constitutive nature of this promoter under high and low concentrations of copper (Theisen *et al.*, 2005).

In methanotrophs, 'copper-switch' is a term often used to depict the concomitant switch in expression from pMMO to sMMO under copper limiting conditions (Stanley *et al.*, 1983). The data presented in Chapter 5 on the transcriptional analysis of the promoters responsible for initiating transcription of the pMMO and sMMO operons gave crucial insights into the molecular regulation of MMO expression and the 'copper-switch'. The data presented in this thesis indicated that a common 'copper-switch' did not regulate the expression of MMO by an on/off switch. Transcription from the pMMO σ^{70} promoter was shown to be constitutive. This was indicated by *Mc. capsulatus* reporter strain carrying a plasmid containing a *gfp* gene fused to pMMO σ^{70} promoter. Furthermore, RNA dot blotting experiments demonstrated that the relative abundance of *pmoA* transcripts were similar in *Ms. sporium* and *Mc. capsulatus* grown under high and low copper conditions. Although pMMO activity was not monitored in this study under varying copper concentrations, extensive fermentation studies carried out by S. D. Prior (Prior, 1985) demonstrated the parallel expression of pMMO and sMMO under low copper conditions where pMMO activity was shown to decrease with increasing sMMO activity (Figure 7.1). This supports the transcriptional data presented here that indicates transcription of the pMMO operon is not repressed under low copper conditions as proposed in an earlier

hypothetical model of MMO regulation (Figure 1.8). These data were consistent with the observations in sMMO-minus mutants where pMMO alone was able to sustain growth under low copper growth conditions.

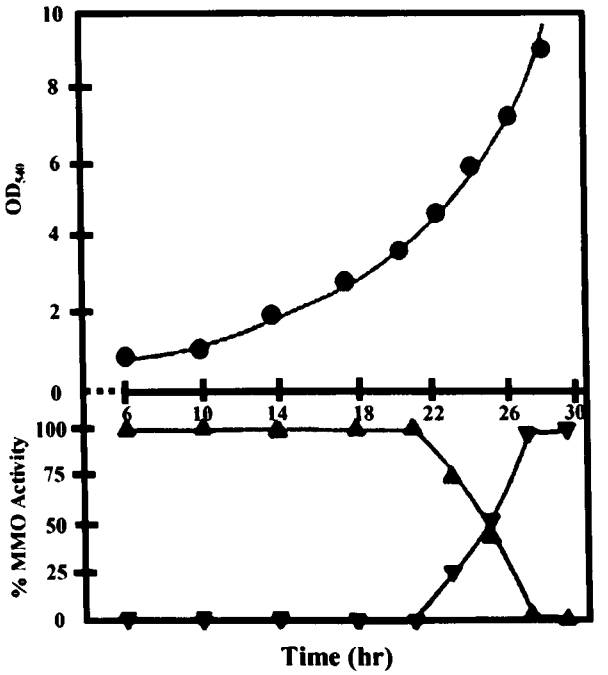

Figure 7.1 Batch growth of *Mc. capsulatus* on low (0.2 mg/l $CuSO_4$) copper containing medium. pMMO (▲) and sMMO (▼) activity was determined by measuring the rate of propylene epoxidation. Figure taken from PhD thesis of S. D. Prior (Prior, 1985).

Based on this information and as discussed in Chapter 5, it can be concluded that the regulation of the pMMO enzyme is at a post-transcriptional level and is independent of the expression of the sMMO enzyme.

Expression of the sMMO enzyme was demonstrated to be tightly regulated at the transcriptional level by a unique 'copper-switch', where transcription from the sMMO σ^{54} promoter was only initiated under low copper growth conditions. In addition, the regulatory genes, *mmoR* and *mmoG* were shown to be essential for transcriptional initiation of the sMMO genes. Moreover, transcription of *mmoR* and *mmoG* was shown to be copper-independent. However, it can not be ruled out that MmoR and MmoG are regulated post-transcriptionally or by an unidentified copper-containing protein. The data gained in this study allows an improved model for the regulation of MMO expression in *Mc. capsulatus* to be proposed (Figure 7.2.).

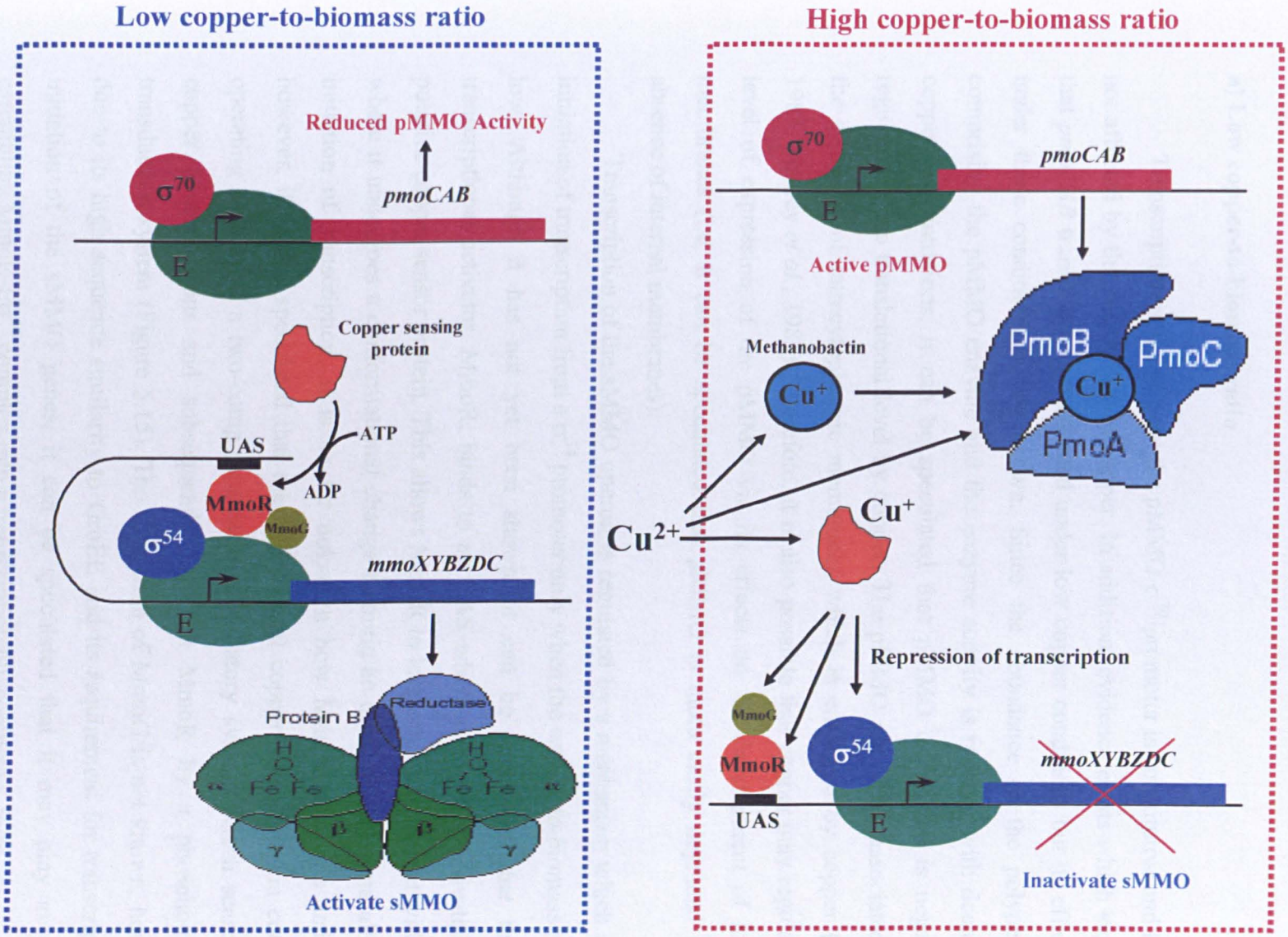

Figure 7.2 A hypothetical model for MMO regulation by copper. Abbreviation: E, RNA polymerase holoenzyme; UAS, Upstream activating sequence See text for description of model.

The mechanism of MMO regulation based on the proposed hypothetical model can be described as follows:

a) Low copper-to-biomass ratio

Transcription initiated from the pMMO σ^{70} promoter is constitutive and thus is not affected by the availability of copper. In addition, evidence exists which suggests that *pmoCAB* transcripts are translated under low copper conditions but its efficiency under these conditions is not known. Since the abundance of the polypeptides comprising the pMMO enzyme and the enzyme activity is reduced with decreasing copper concentrations, it can be speculated that pMMO expression is negatively regulated at the translational level by copper. The pMMO activity is associated with the synthesis of intracytoplasmic membranes which is enhanced by copper (Prior, 1985; Stanley *et al.*, 1983). Therefore, it is also possible that copper may regulate the level of expression of the pMMO via its effects on the development of internal membranes (i.e. it can be speculated that pMMO is more easily degraded in the absence of internal membranes).

Transcription of the sMMO operon is regulated by a mechanism which allows initiation of transcription from a σ^{54} promoter only when the copper-to-biomass ratio is low. Although it has not yet been shown, it can be speculated that the σ^{54} transcriptional activator, MmoR, binds to an UAS where it becomes activated by a putative copper sensor protein. This allows MmoR to loop over and interact with $E\sigma^{54}$ where it undergoes a conformational change forming an open complex and allowing initiation of transcription. It is so far unknown how MmoR might be activated, however, it can be speculated that the hypothetical copper sensor protein could be operating as part of a two-component sensor regulatory system, which senses low copper concentrations and subsequently activates MmoR by a phosphotransfer transduction system (Figure 5.15). The exact role of MmoG is not known, however, due to its high sequence similarity to GroEL and its requirement for transcriptional initiation of the sMMO genes, it can be speculated that it may play a role in assembling MmoR-$E\sigma^{54}$ complex into a transcriptionally competent form.

b) High copper-to-biomass ratio

Expression of pMMO and the production of intracytoplasmic membranes are stimulated by copper (Nguyen *et al.*, 1994; Stanley *et al.*, 1983). The mechanism involving the delivery of copper to pMMO is not known, however, a recent study has exclusively shown association of the copper-containing compound, methanobactin to pMMO activity (Kim *et al.*, 2004). Additionally, there are many putative copper uptake proteins identified in *Mc. capsulatus* and therefore there may be other unidentified copper delivery systems for pMMO.

It is well established now that transcription from the sMMO σ^{54} promoter is totally repressed by copper and it can be speculated that it could be a result of the inability of the putative copper sensing protein to activate MmoR due its repression directly by copper. Currently there is no evidence to suggest that MmoR or MmoG could be directly repressed by copper since they lack copper binding motifs (Csaki *et al.*, 2003; Stafford *et al.*, 2003). Furthermore, it is also possible that transcription could be prevented by a copper-containing repressor protein, which is interacting with a) MmoR leading to the formation of an inhibitory protein-protein complex, b) MmoG thus preventing it from assembling a competent MmoR-Eσ^{54} complex or c) it may be competing with MmoR for the UAS. The repression of transcription through inactivation of the σ^{54}-dependent transcriptional activator, MmoR, by a repressor protein can be supported by an analogous system in *Azotobacter vinelandii* for regulating the conformational switch involved in nitrogen fixation. In this system the nitrogen fixing genes, *nif*, are regulated at the transcriptional level by the σ^{54}-dependent transcriptional activator, NifA, and an anti-activator, NifL. The NifL regulates the activity of NifA in response to the redox, carbon and nitrogen status and is regulated itself by signal transduction protein, GlnK, which becomes covalently modified by uridylylation in response to nitrogen deficiency (Little *et al.*, 2006).

In methanotrophs, a common mechanism seems to be regulating the expression of MMO by copper, with the exception of *M. silvestris*, where a BHR promoter probe vector was used to obtained data suggesting the constitutive nature of the sMMO σ^{54} promoter. Interestingly, in this methanotroph, the regulatory genes *mmoR* and *mmoG* were found to be co-transcribed with the sMMO structural genes, which is the operon they hypothetically regulate and indicated a potentially alternative mode of regulation of sMMO expression (Theisen *et al.*, 2005). In an experiment by Syed and Gralla,

(1997) it was demonstrated, through site-directed mutagenesis that a number of leucines found in the N-terminal region of σ^{54} (RpoN) have a role in keeping unregulated transcription in check. A number of mutations in these leucines allowed leaky transcription to occur *in-vivo* in the absence of activator protein (Syed & Gralla, 1997). In light of this information, it can be speculated that a similar mutated σ^{54} protein may be present in *M. silvestris* which allows leaky transcription of the sMMO operon from the σ^{54} promoter in the absence of MmoR and MmoG. Further investigations along these lines might also help to explain the constitutive nature of this promoter.

To understand the exact mechanism of the copper mediated regulation of MMO expression, additional regulatory components, such as anti-activators/repressors need to be identified. As it can be seen from the transcriptional regulation of nitrogen fixation in *Azotobacter vinelandii*, as described above, the regulatory systems of σ^{54}-depedent transcription is complex and often involves several cascades of regulatory components. Therefore, identification of such regulatory components is not trivial and to this end a functional genomics approach for the genome-wide analysis was also taken in this study. This included establishing a transposon mutagenesis protocol in *Mc. capsulatus* from which a number of sMMO-minus mutants were identified (as discussed in Chapter 6). Transposon mutagenesis, despite being a powerful tool, has a few limitations and owing to the simple detection of β-galactosidase activity using the MUG reagent and its high-throughput nature, a modified genetic system incorporating *Mc. capsulatus* LacZ reporter strain and transposon mutagenesis was developed. The simplicity of this system as a high-throughput screening method for the rapid detection of sMMO minus mutants resulting from inactivation of genes involved in transcription initiated from sMMO σ^{54} promoter was demonstrated through the identification of three LacZ minus mutants. This also highlighted a minor limitation of this system due to the specificity of Tn*5* for LacZ, however, with some minor modification to the transposon vector, as suggested in Chapter 5, this system looks very promising for identifying the key regulatory genes involved in the transcriptional regulation of sMMO. This system can also be extended to other applications, for example, looking at the post-transcriptional regulation of the pMMO enzyme by creating translational fusions with the pMMO σ^{70} promoter and LacZ and subsequently using the reporter strain for transposon mutagenesis.

In addition to the above approaches, a directed evolution approach using EP-PCR was also taken to identify variant promoters with altered activity and UAS where MmoR binds. The amplification of sMMO σ^{54} promoter with varying frequency of mutations fused to LacZ yielded a powerful tool for carrying out detail promoter analysis in methanotrophs. This technique can be extended further by developing a system which will allow transcription to initiate from the sMMO σ^{54} promoter in *E. coli* and allow the subsequent rapid analysis of mutated copies of sMMO σ^{54} promoter. Development of such a system will also allow mutational analysis to be performed on the regulatory genes, *mmoR* and *mmoG* which will provide detailed information on their mode of regulation.

Overall, the work carried out in this study has given further insights into the molecular regulation of methane monooxygenase by copper. This will no doubt serve as a good platform for future researchers to further exploit this information and together with the availability of a wide range of genetic tools developed in this study, will help solve the long-standing quest for elucidating the molecular regulation of MMO in methanotrophs.

7.2 Future work

A number of questions still remain unanswered, particularly the exact molecular mechanism regulating the tight transcriptional regulation of sMMO expression and the post-transcriptional mechanisms regulating pMMO activity. The short term experimental approaches which can be taken have been discussed in detail within each chapter and also above.

In this study, a molecular approach was taken to gain a deeper understanding of the transcriptional regulation of MMO expression by copper. To gain further detailed understanding of MMO regulation leading to a greater ability to control the expression of MMO enzyme in the environment and in an industrial setting, a biochemical approach, in addition to the molecular approach, will be required to elucidate and identify other, as of yet undiscovered components involved in the regulation such as:

- Regulatory proteins involved in the copper-regulated expression of MMO

- Proteins/chaperonins involved in the insertion of metal centers and assembly of sMMO and pMMO into functionally active complexes

- Proteins involved in sensing copper levels and the subsequent sequestering and transportation of copper to target proteins such as pMMO and copper-containing repressor proteins involved in the transcriptional regulation of sMMO

The initial way forward to identifying candidate genes involved in the regulation of MMO expression and genes encoding for copper sensing and transport proteins, would be to exploit the genetic screen, based on *lacZ* reporter gene and transposon mutagenesis, developed in this study. Such an approach would allow for the rapid screening of regulatory genes on a global scale.

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